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Induction of Regeneration of Forelimb of the Frog by Augmentation of the Nerve Supply.* (18508)

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The ability to regenerate extremities following amputation is observed in all stages of the life cycle of the salamander and other urodele amphibians but, in the case of frogs, it is restricted mainly to the tadpole (Barfurth, review by Polejaiev) (1,2) although occasionally a limited regeneration is seen in the adult (2,3). The loss in regenerative capacity occurs rather abruptly during metamorphosis (2), but it is not an absolute and irrevocable one for regeneration has been successfully evoked in limbs in which the wound area was subjected to mechanical and chemical irritations and alterations (2,4-8). The reason for the decline in regenerative power at metamorphosis is not known but a number of theories have been advanced which are re-

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2. Polejaiev, L. W., *Arch. d'Anat. Micr.*, 1936, v32, 437.
3. Thornton, C. S., and Shields, T. W., *Copeia*, 1945, v40.
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viewed and evaluated by Polejaiev(2,5), Rose (7,9) and others. Salient among these views is the belief that some fundamental change occurs in the tissues or tissue interrelations which render extremities incapable of regeneration. The nature of the change has been speculated upon and the idea has been advanced that it involves increased "differentiation" or alteration in the "organizing power" of the limb. These theoretical explanations of regenerative loss in the adult frog must at this time be supplemented or, perhaps, partly substituted by another theory which has emerged from the results of experiments on the influence of the nerve in regeneration of the forelimb of the newt (Singer)(10-12).

In the newt, regeneration of the forelimb does not ensue if the number of nerve fibers normally available at the amputation surface is reduced beyond a critical threshold value through partial destruction of the contributing brachial nerves. Perhaps such quantitative requirements obtain also for the tadpole limb since it has been shown in experiments on the tadpole that nerves are essential for regeneration(13). Assuming that such threshold requirements do, indeed, exist, it is possible to advance the theory that loss in regenerative capacity of the frog's limb which occurs during metamorphosis is due, at least in part, to tissue or other changes which render the available nerve fibers numerically inadequate to evoke regeneration. If such changes do occur whereby the threshold nerve requirements for initiation of regeneration are no longer met by the existing nerve supply, then it would follow that regeneration should ensue if such threshold nerve requirements are satisfied by some experimental means. In experiments described here the normal nerve supply of the forelimb was augmented substantially by nerves deviated from the hindlimb. As the results demonstrate, regeneration of the forelimb of the post-metamorphic frog is readily evoked thereby.†

Procedures. Young adult frogs (*R. pipiens* —about 3 cm body length) were anaesthetized with ether and subjected to the nerve operation diagrammed in Fig. 1. The forelimb was amputated in about the middle of the upper arm. Then a slit was made in the skin of the thigh and a threaded needle drawn under the skin of thigh, body wall and upper arm until it emerged at the amputation surface (Fig. 1A). The sciatic nerve and its four major branches were freed by dissection to the level of the ankle or foot and fastened to the end of the thread as shown in Fig. 1B. The free end of the thread was seized and pulled until the remaining thread and attached nerves were drawn under the skin to the amputation surface of the forelimb (Fig. 1C). The distal ends of the nerves were resected where they emerged at the forelimb thus freeing them from the thread. The dissected and nerveless hindlimb was then amputated near the body wall and the skin sutured over its surface. The deviated nerves consisted of the sciatic, its two major branches the tibial and peroneal, and their two subdivisions, respectively the deep and superficial (sural) tibial and the lateral and medial peroneal nerves. Dissection of the latter four subdivisions was carried to the ankle or foot region in order to obtain a sufficient length of nerve for deviation. Deviation of the nerves was done in 21 animals. Nineteen others of the same stock were used as controls. In all controls the amputation level of the forelimb was the same as for the experimental ones. In 9 control animals the hindlimb of the same side was also amputated and the skin sutured over the cut surface but this variation yielded no difference in results and is, therefore, not mentioned further. Reference will also be made in the ensuing description to other experimental animals which were employed in the preliminary elaboration and survey of the experiments

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11. Singer, M., *J. Exp. Zool.*, 1947, v104, 223.

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† These theoretical interpretations as well as initial successful results with two animals were presented by the author for the first time in the discussion of a paper given by Rose at the AAAS meetings in Boston in 1946. They are mentioned by Rose in his later review(9).

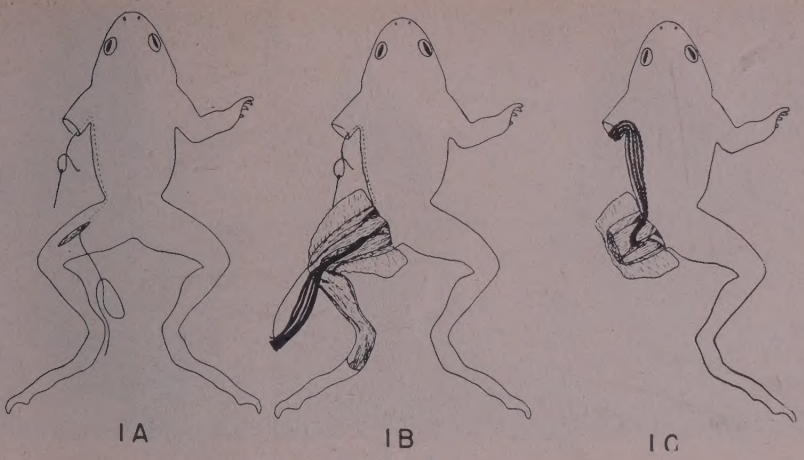

FIG. 1A, B, C.
Diagram of the successive operative procedures used in deviating the sciatic nerve and its branches to the amputation surface of the forelimb.

described herewith. All operations were confined to the left side. Animals were kept in aquarium tanks containing dilute NaCl solution (0.15%) rather than tap water to avoid infections and the tanks were tilted slightly to provide an amphibious environment. Animals were force-fed 2 or 3 times a week with ground beef dipped in cod liver oil and bone meal according to the recommendation of Rose(7). The amputated forelimb was observed periodically for signs of regeneration.

Results. Three of 19 control animals showed some limited signs of forelimb regeneration after 6 to 8 weeks. These growths were quite small (approximately 0.75 mm long in one case and less than 0.50 mm in the other 2), appeared later than those described for experimental animals and were restricted to a small part of the amputation surface. The appearance of an occasional regenerate among the controls emphasizes the fact that regenerative capacities of the post-metamorphic frog are latent. Signs of occasional spontaneous regeneration, particularly of more distal levels of the frog's limb, have been observed before(2,3).

Twenty of the 21 animals in which nerves were deviated showed a regenerative response of the forelimb, and the regenerate in 16 of these cases was larger than in the positive control ones. At 8 weeks following operation most of these 16 were between 2 and 3 mm in length and continuing to grow. Initial experiments of nerve deviation with other animals showed that lengths of 6 mm or more could be eventually attained. Generally, the first gross signs of regeneration appeared about 3 weeks after amputation but in some instances as early as 2 weeks. The rate of growth was greatest in the period immediately following growth initiation.

The first signs of morphogenesis appeared 8 to 10 weeks after operation and subsequently showed tremendous variations among the animals in extent and rate. In some instances a bend appeared in the regenerate suggesting elbow formation; or, a distal flattening occurred which was reminiscent of hand formation as seen in newt regeneration. Another sign of differentiation was the appearance of irregularities over the distal end of the regenerate which anticipated finger formation. Examples of regenerates in various conditions of differentiation are shown in Fig. 2 and 3, which may be contrasted with the non-regenerating control of Fig. 4. In general, regenerates were quite heteromorphic as compared to those of the salamander.

Finally, preliminary observations of histological sections through regenerates revealed extensive histogenesis of limb tissues. Cartilaginous skeletal elements having articulating

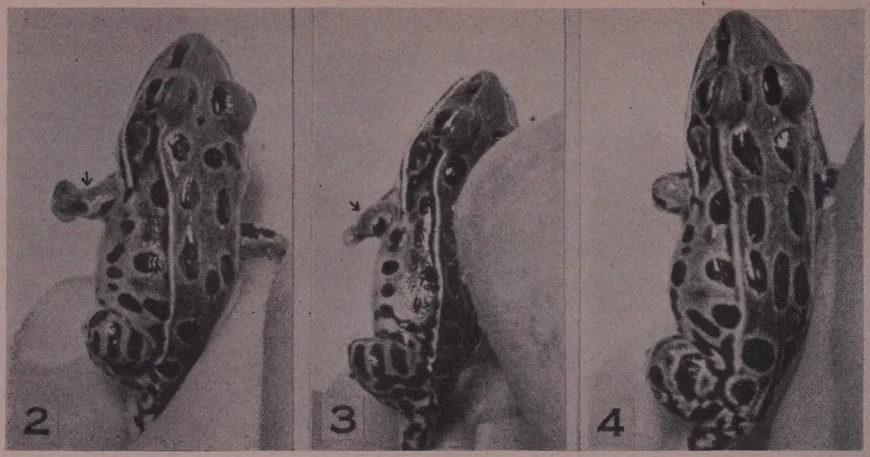

FIG. 2, 3, 4.
Representative results of experiments. Fig. 2 and 3 show regenerates of 8 weeks, stimulated by the augmented nerve supply, whereas Fig. 4 shows a non-regenerating control. Arrows in Fig. 2 and 3 point to original amputation level. (1½×).

surfaces, muscle, tendon and other connective tissue elements were present in various stages of development.

Discussion. The results show that regeneration of the forelimb of the frog may be successfully evoked by an augmented nerve supply. It is reasonable to conclude therefrom that the nerves normally available at the amputation surface are quantitatively inadequate to yield a regenerative response. Alternative explanations seem less likely; for example, that the sciatic nerve possesses the peculiar quality of stimulating forelimb regeneration not shared by nerves of the forelimb, or that potentialities of growth stimulation, latent within the nerve, are somehow called forth when the nerve is deviated to the forelimb. Assuming that the nerve action is non-specific and the essential effect of nerve deviation was to raise the fiber quantity above the threshold, then it is evident that loss of regenerative capacity which occurs at metamorphosis is the result of some anatomical or physiological change which renders the available nerve fibers quantitatively an ineffective stimulus of regeneration. Theoretically the anatomical or physiological change may have its locus in the nerve, in other tissues of the forelimb, or, indeed, in both.

These results re-emphasize an aspect of nerve function which is little appreciated, namely the ability to stimulate growth of another structure(14). As shown experimentally here, this quality of the nerve is not lost at the time of metamorphosis but is retained in the adult and under appropriate circumstances may be expressed in organ regeneration.

Summary. The ability of the amputated forelimb of the tadpole to regenerate declines and in most cases is lost completely at the time of metamorphosis. However, the present report shows that regeneration can be evoked experimentally in the adult frog if the number of nerve fibers available at the amputation surface of the forelimb is augmented by deviation of the sciatic nerve from the hindlimb. The theoretical considerations which led to these experiments emerged from the study of the influence of quantity of nerve fibers in regeneration in salamanders and are discussed here.

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Replacement of Pteroylglutamic Acid by Folinic Acid for the Chick.* (18509)

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The synthesis of a compound related to, but not identical with, pteroylglutamic acid (PGA) has been reported by Shive *et al.*(1). The compound(2) is synthesized by hydrogenation of formylfolic acid. It contains 4 more hydrogen atoms and appears to be identical with the naturally-occurring folinic acid, as described by Bond *et al.*(3). Both the natural folinic acid(4) and the synthetic folinic acid(1) have been shown to satisfy the nutritional requirements of *Leuconostoc citrovorum* 8081 for a factor described by Sauberlich and Baumann(5). An excellent review of the subject has recently been published(6). Broquist *et al.*(7) reported that concentrates of the *L. citrovorum* factor replaced PGA for the chick. The synthesis and isolation of a crystalline material which is active in place of the *L. citrovorum* factor for this organism was recently announced by Brockman *et al.* (8). These authors also stated that this material was active for the chick in place of PGA, but gave no data.

In the light of this evidence, it was of interest to determine whether the synthetic folinic acid is physiologically active for the chick in place of PGA.

Experimental. Day-old White Leghorn male chicks from the Poultry Division flock were used. They were sexed at time of hatching and raised in electrically-heated, metal brooders with wire screen floors. Feed was supplied *ad libitum* and the water changed daily. Six chicks were used per group and the experiments were terminated at the end of 4 weeks. The basal ration (SIF), low in PGA, consisted of 56.5% cerelose, 20% casein, 8% gelatin, 5% cellulose, 4% soybean oil, 6% salts 2M†, 0.3% DL-methionine, 0.2% choline chloride, and the following vitamins‡ (mg/kg): thiamine hydrochloride, 4; riboflavin, 8; calcium pantothenate, 20; pyridoxine hydrochloride, 6; nicotinic acid, 100; biotin, 0.2; α-tocopherol acetate, 5; and 2-methyl 1,4-naphthoquinone, 1. To each kg of diet were added 10,000 I. U. of vit. A and 1000 A.O.A.C. units of vit. D_3. The synthetic folinic acid used in these trials was prepared and supplied by Dr. William Shive of the University of Texas.

Results. The results are shown in Table I. In the first trial the addition of 0.5 mg of folinic acid/kg of ration gave a growth response equal to that of an equal amount of PGA. The feed efficiency figures were also similar. That 0.5 mg of folinic acid/kg or 0.5 mg of PGA was probably not adequate in this ration is shown by the difference in weights of these groups and the group receiving 2 mg of PGA/kg. In the first trial the basal ration contained no added vit. B_{12},

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† Salts 2M(g/600 g): $CaCO_3$-150, K_2HPO_4-90, Na_2HPO_4-73, $Ca_3(PO_4)_2$-140, NaCl-88, $MgSO_4 \cdot 7H_2O$-50, ferric ammonium citrate (17-18% iron)-4, $MnSO_4 \cdot 4H_2O$-4.2, KI-0.4, $ZnCl_2$-0.2, and $CuSO_4 \cdot 5H_2O$-0.2.

‡ We are indebted to Merck and Co., Rahway, N. J., for crystalline vitamins, Lederle Laboratories, Pearl River, N. Y., for the pteroylglutamic acid, and to U. S. Industrial Chemicals, Inc., N. Y., for DL-methionine.

TABLE I. Studies with Synthetic Folinic Acid.

Trial	Group	Supplement/kg of basal ration SIF	Avg wt, g, 4 wk	Feed efficiency*
1	1	0	120	2.63
	2	.5 mg folinic acid	226	2.01
	3	.5 mg PGA	231	1.96
	4	30 mcg cryst. vit. B_{12}	127	2.51
	5	1 mg cryst. vit. B_{12}	142	2.33
	6	2 mg PGA	269	2.13
			26 days	
2†	1	0	106	2.66
	2	.1 mg folinic acid	126	2.57
	3	.1 mg PGA	105	3.08
	4	.4 mg folinic acid	209	2.09
	5	.4 mg PGA	167	2.21
	6	2 mg PGA	211	2.13

* Feed efficiency—Total feed consumed / wt gained

† Trial 2—30 mcg cryst. vit. B_{12}/kg added to basal ration.

which has been reported to spare PGA(9).

In trial 2, two different levels of folinic acid were compared to similar levels of PGA. The growth responses and feed efficiency figures show that the folinic acid was fully as active as PGA; whether it may be even more active will require more work to fully determine.

It is clearly evident that folinic acid should be added to the list of known compounds of the folic acid group which are active for the chick.

Summary. Studies on the biological activity of synthetic folinic acid show that it may replace pteroylglutamic acid in the diet of the chick.

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Effect of Administration of ACTH and Cortisone upon Blood Glutathione Levels (18510)

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Conn and his associates(1,2) reported that the administration of ACTH resulted in diminished glucose tolerance and glycosuria which were associated with a decrease of blood glutathione (GSH) and an increased excretion of urinary uric acid. Conn, Lewis, and Johnston(3) showed that the administration of GSH to a normal individual receiving ACTH produced a dramatic but transitory reduction in the hyperglycemia and glycosuria. This observation, as Conn *et al.*(1) have indicated, is of interest inasmuch as the diabetogenic activity of alloxan, a possible purine metabolite, can be decreased by GSH as well as other substances containing the thiol group. Kinsell *et al.*(4) found an increased urinary excretion of sulfur-containing compounds following the administration of ACTH. Lazarow and Berman(5) noted that the injection of cortisone into normal rats led to a decrease in blood GSH which appeared to correlate with the degree of glycosuria. Lazarow (6) found that GSH potentiated the action of cortisone and, in contrast to its action during ACTH therapy reported by Conn *et al.* (3), produced an increased glycosuria when injected into cortisone diabetic rats.

The observations of Conn *et al.*(1,2) upon

* Lt. j.g. (M.C.) U.S.N.

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2. Conn, J. W., Louis, L. H., and Johnston, M. W., *J. Lab. and Clin. Med.*, 1949, v34, 255.

3. Conn, J. W., Louis, L. H., and Johnston, M. W., *J. Clin. Invest.*, 1949, v28, 775.

4. Kinsell, L. W., Li, C. H., Margen, S., Michaels, C. D., and Hedges, R. N., Proc. of the First ACTH Conference, Blakiston Co., Phila., 1950, p. 70.

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6. Lazarow, A., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 702.

TABLE I. Blood Glutathione (GSH) and Total Glutathione (GSH and GSSH) and Blood Glucose Following ACTH.

No.	Control	mg %	During administration of ACTH mg %	mg %	mg %	mg %	Max. reduction in GSH, %
1	GSH	40.9	*37.3*(2)*	42.4(5)	39.3(7)	37.8(10)	9
	Total	50.8	53	47.1	56.3	54.6	
	Glucose	95	106		112		
2	GSH	47.0	47.1(1)	46.2(2)	*24.6*(6)	35.4(8)	48
	Total	63.2	64.7	60.1	62.1	53.9	
	Glucose	91		131	106	103	
3	GSH	33.9	*24.6*(1)	27.7(5)	27.7(8)	39.8(11)	28
	Total	47.6	33.9	43.1	38.5	45.6	
	Glucose	95	97	106	94	95	
4	GSH	44.6	33.9(1)	32.3(2)	*27.7*(4)	38.5(6)	39
	Total	48.0	43.1	43.1	42.0	46.2	
5	GSH	43.1	40.0(2)	43.1(4)	*31.6*(8)	46.2(18)	26
	Total	46.2	67.8	67.8	70.8	55.4	
	Glucose	95		105		105	
6	GSH	32.3	*30.8*(2)	38.6(3)	43.1(5)	40.0(9)	5
	Total	53.3	47.7	46.2	52.4	46.2	
	Glucose	100	107	96	112	95	
7†	GSH	33.1	24.6(2)	*20.0*(5)	26.1(10)	26.1(15)	40
	Total	40.8	38.5	37.0	37.0	35.4	
	Glucose	113	126	138		128	
8	GSH	34.0	*26.2*(1)	34.5(3)	38.5(5)	33.4(7)	23
	Total	56.2	45.6	44.6	52.4	52.4	
	Glucose	102	102	107	95	96	
9	GSH	31.4	34.4(1)	31.4(4)	*23.6*(6)	28.3(8)	25
	Total	44.0	45.6	43.1	38.6	46.3	
	Glucose	97		114	100		
10	GSH	32.2	*22.0*(1)	25.1(3)	34.9(4)		32
	Total	46.3	40.0	41.1	45.0		
	Glucose	99	117	104			
11†	GSH	30.4	*21.6*(2)	21.6(4)	33.9(7)	27.7(9)	29
	Total	50.0	55.0	40.0	46.2	46.2	
	Glucose	97	129	121	109	115	
Avg	GSH	36.6 ± 6.0		26.8 ± 5.5		t = 4.23	
	Total	49.7 ± 6.0		47.9 ± 11.4		t = 0.50	

* No. in parentheses = No. of days on ACTH.
† Glycosuria developed on therapy.

the lowering of blood GSH levels following the injection of ACTH were made upon four patients and have been confirmed, in one case, by Kass, Ingbar, and Finland(7). Sprague *et al.*(8) followed the blood GSH in three patients who received both ACTH and cortisone and although the GSH showed little alteration with ACTH a slight increase in concentration of GSH occurred during cortisone therapy. We have determined GSH and total glutathione (GSH plus GSSG) in the blood following the administration of both ACTH and cortisone and have also determined blood sugar quantitatively and urine sugar qualitatively.

Experimental. Reduced glutathione (GSH) and total glutathione (GSH plus GSSG) were determined by the iodometric method of Hess (9) as modified by Woodward and Fry(10). This method has been criticized for a lack of specificity because both ergothioneine and ascorbic acid will react with iodine. As Hess (9) pointed out ergothioneine, present in

7. Kass, E. H., Ingbar, S. H., and Finland, M., PROC. SOC. EXP. BIOL. AND MED., 1950, v73, 669.

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9. Hess, W. C., *J. Wash. Acad. Sci.*, 1929, v19, 419.

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TABLE II. Blood Glutathione (GSH) and Total Glutathione (GSH and GSSH) and Blood Glucose Following Cortisone.

No.	Control	mg %	During administration of cortisone mg %	mg %	mg %	mg %	Max. reduction in GSH, %
1	GSH	30.5	34.1(3)*	*25.1*(10)			18
	Total	46.1					
	Glucose	85					
2	GSH	27.8	*15.7*(2)	22.9(7)			43
	Total	48.6	39.3	47.7			
	Glucose	115	114	110			
3	GSH	33.0	19.8(1)	*18.8*(4)	34.4(11)		40
	Total	55.4	52.8	41.8	45.6		
	Glucose	105		98	114		
4	GSH	34.9	*26.7*(7)				20
	Total	41.9	43.0				
5	GSH	47.1	40.4(1)	*36.9*(2)	42.4(3)	44.0(5)	21
	Total	59.9	51.1	49.1	51.9	54.6	
	Glucose	97		104	102	106	
6	GSH	42.3	44.7(2)	30.8(6)	*27.7*(9)	30.8(18)	34
	Total	61.8	58.5	49.3	50.8	48.3	
	Glucose	90		89			
7	GSH	40.0	47.7(4)	*38.5*(5)			4
	Total	61.6	55.4	61.6			
	Glucose		107	110			
8	GSH	50.8	53.9(1)	*46.2*(3)	57.0(7)	55.4(10)	9
	Total	63.1	66.2	67.8	72.6	68.3	
	Glucose	101		91			
9	GSH	26.2	44.7(1)	*35.4*(6)			(increase = +35)
	Total	47.7	59.2	55.0			
	Glucose	95	107	99			
10	GSH	39.3	*35.5*(1)	43.3(2)			10
	Total	52.3	44.5	56.6			
	Glucose	120	110	95			
11	GSH	28.6	36.9(1)	37.2(2)	*31.4*(3)	37.4(4)	(increase = +8)
	Total	42.3	47.1	45.7	51.2	48.6	
	Glucose	100	100	92	95		
12	GSH	19.3	*11.7*(3)	18.3(7)	29.7(15)		39
	Total	33.7	41.0	48.8	44.6		
	Glucose	102	103				
Avg	GSH	35.0 ± 9.0		29.1 ± 11.1	t = 1.42		
	Total	51.2 ± 9.9		48.9 ± 8.8	t = 0.62		

* No. in parentheses = No. of days on cortisone.

whole blood to the extent of only 7-8 mg per 100 cc, has a titer in this method of less than one-tenth of that of an equal weight of GSH and, consequently, cannot cause any significant error. The amount of ascorbic acid present in blood likewise is too small to affect the titer for GSH. The more specific amperometric method of Benesch and Benesch(11), as pointed out by them, gives values for blood GSH which are the same as those obtained by the method we have employed. ACTH and cortisone were administered in amounts that gave the usual physiological responses, eosinopenia, increased urinary excretion of 17 ketosteroids and so forth.

The changes which occurred with ACTH are given in Table I and those with cortisone in Table II. Blood glucose values are included in both tables. The control values are, in almost every instance, the average of two or more determinations upon fasting blood samples. The average GSH values for the control periods are substantially the same in both groups. The figure in parenthesis following each of the values obtained during the experimental periods is the number of days under treatment. The lowest GSH value

11. Benesch, R. R., and Benesch, R., *Arch. Biochem.*, 1950, v28, 43.

obtained in each series of determinations during treatment is italicized and the average GSH and total glutathione values together with their standard deviations are calculated from these data.

Following the administration of cortisone there was a drop in GSH to 29.1 mg per 100 cc of blood from the control value of 35.0 mg. However, the t value was 1.42 indicating that the decrease had a low order of significance. The administration of ACTH produced a more marked lowering of the GSH from the control value of 36.6 mg per 100 cc of whole blood to a value of 26.8 mg. There was no appreciable change in the total glutathione value after the administration of either ACTH or cortisone indicating that the decrease in the GSH was brought about mainly by its oxidation.

The blood sugar increased more than 10 mg per 100 cc of blood over the control values in eight of ten patients following the administration of ACTH. In three patients the highest blood sugar level occurred on the same day as the lowest GSH value and the highest figures for the other five patients were obtained, on the average, 1.2 days following the lowest GSH value. One patient, not included among those just discussed, with hyperinsulinism caused by hypertrophy of the islet cells (number 4 in Table I) had a control blood sugar level of 37 mg per 100 cc of blood which rose to 93 mg after four days of ACTH therapy. On this day the GSH concentration had dropped to 27.7 mg per 100 cc of blood from an initial value of 44.6 mg. After the discontinuance of treatment the GSH concentration returned to 38.5 mg per 100 cc and the blood sugar decreased to 33 mg. Following the administration of cortisone only two of eleven patients showed an increase in blood sugar of more than 10 mg per 100 cc over the control values. One of these demonstrated the highest blood sugar on the same day as the lowest GSH value but the other had the most marked hyperglycemia five days before the lowest GSH level was attained.

Glucose tolerance tests were conducted on four patients before and during ACTH therapy. Two of these patients had mild diabetic patterns prior to ACTH administration but showed no further impairment of carbohydrate tolerance during therapy. The average decrease in their blood GSH was 16 percent. The other two patients developed typical diabetic glucose tolerance curves during ACTH therapy whereas their curves were normal prior to treatment. The average decrease in blood GSH was 38 percent. The number of patients is too small to draw any conclusion but the results do indicate that the change in glucose tolerance and GSH may be related. They also suggest, contrary to expressed opinion(8), that although individuals with normal glucose tolerance may show impairment of carbohydrate metabolism during adrenal stimulation those with mildly decreased tolerance demonstrate no further inability to handle glucose.

Glucose tolerance tests were conducted on four patients receiving cortisone. In two cases the curves were mildly diabetic before therapy and showed no change during treatment, the decrease in GSH averaging 5.5 percent. In one patient the curve was normal before cortisone but became typically diabetic during treatment and the GSH decreased 14 percent. The last patient had normal glucose tolerance curves before and during cortisone therapy and the blood GSH actually increased 35 percent.

A further indication of the relationship between the blood glucose level and the decrease in blood GSH following ACTH may be noted by correlating the increase in blood glucose with the decrease in GSH. As shown in Table I the five subjects with the greatest increase in blood sugar following ACTH therapy showed an average decrease in GSH of 34.8 percent whereas the five subjects with the least increase in blood glucose gave an average decrease in GSH of only 19 percent.

Summary. Blood glutathione (GSH) and total glutathione (GSH plus GSSG) were determined in eleven patients during ACTH therapy and in eleven patients during the administration of cortisone. GSH values were significantly decreased from a pretreatment average of 36.6 mg per 100 cc to 26.9 mg by ACTH while cortisone produced a decrease

from an average pretreatment value of 35.0 mg per 100 cc to 29.1 mg. The latter decrease, statistically, is of a low order of significance. The total glutathione values showed no changes following the administration of either ACTH or cortisone. During treatment with ACTH the greatest decrease in blood GSH occurred in the patients showing the greatest increase in blood sugar. There is suggestive evidence that although patients with normal glucose tolerance may show diabetic patterns during treatment, those who demonstrate mildly diabetic patterns prior to therapy show no further decrease in glucose tolerance and minimal reduction in GSH values after ACTH or cortisone therapy.

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Inhibition of Citric Acid Synthesis *in vivo* by X-irradiation.* (18511)

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Previous investigations(1-4) have shown that the activity of a number of enzymes is markedly inhibited by ionizing radiations *in vitro*. However, the extent to which enzyme inactivation is involved in the acute toxic effects of ionizing radiations has not yet been clearly established. Thus, further studies on the biochemical changes in tissues taken from irradiated animals are indicated in order to find which enzymatic reactions are affected by ionizing radiations in the intact animal. The recent introduction by Potter(5) of the concept of sequential blocking of metabolic pathways has provided a new approach for studying the actions of toxic agents on intermediary carbohydrate metabolism *in situ*. This procedure consists of the use of an inhibitor known to block a reaction in the tricarboxylic acid cycle with the resultant accumulation of one of the Krebs intermediates in concentrations high enough to allow quantitative measurements. Sodium monofluoroacetate was employed by Potter(5) for this purpose. Buffa and Peters(6,7) demonstrated that this compound causes an accumulation of citric acid in rat tissues and subsequent investigations(8-11) have shown that this effect is due to inhibition of the oxidation of citric acid probably through prior conversion(8,9,12) of fluoroacetate to fluorocitrate or a related compound. Detailed studies by Potter, Busch, and Bothwell(13) have established standard conditions for use of fluoroacetate as an inhibitor of citrate oxidation *in vivo*.

In the present investigation the technic of sequential blocking(5) was employed to study the effects of X-ray on the operation of the

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citric acid cycle *in vivo* at the suggestion of Dr. Van R. Potter. The standard fluoroacetate treatment(5) was administered to rats at various times after X-ray and citrate accumulation in tissues from normal and irradiated animals was compared. The results indicate that X-irradiation causes a marked inhibition of citrate accumulation in some tissues and an increase in the citrate content of liver from fluoroacetate-treated rats. The magnitude and duration of these effects were found to be dependent upon the dose of X-ray.

Materials and methods. Male Sprague-Dawley rats (175-250 g), adult male Carworth mice and young guinea pigs (200-300 g) were used for these experiments. All doses of X-ray were given in single total-body exposures (250 kv, 15 ma, 0.25 mm Cu filter, 50 cm target distance and 35 to 40 r/min). Groups of 20 mice, 12 rats, or 6 guinea pigs were irradiated simultaneously. Each animal was enclosed in an individual compartment of cylindrical cages with each compartment being equidistant from the center of the field and the cage was rotated continuously. Various doses of X-ray were administered by altering the time of exposure. Aqueous solutions of purified sodium fluoroacetate were given intraperitoneally. Throughout these experiments rats were given 3.5 mg/kg of sodium fluoroacetate and killed 3 hours later (5). Since a few rats succumbed within 3 hours after the fluoroacetate treatment extra animals were included in each experiment to allow for possible early deaths. Guinea pigs received 2 mg/kg and mice were given 10 mg/kg of fluoroacetate intraperitoneally and sacrificed in 3 hours. Species variation necessitated the use of different doses to block citrate oxidation in the 3 species employed. Animals were sacrificed by decapitation and the citrate content of the tissues was measured by the method of Natelson *et al.*(14) using the modifications recommended by Potter and Busch(11).

Results. To ascertain whether X-irradiation affects citrate formation *in vivo* rats were given 800 r of X-ray and groups of animals were then treated with fluoroacetate at various intervals up to 72 hours following irradiation. In 3 hours after the fluoroacetate treatment tissues were removed for citric acid analyses. The results of this experiment are shown in Fig. 1 in which each value represents an average for 6 animals. Marked atrophy of the spleen and thymus made pooling of the tissues from 3 animals desirable for accurate analyses when the animals were sacrificed later than 12 hours after 800 r. Individual variations in the citrate content of the tissues were of the same magnitude as those observed in other investigations(11,13). By the use of groups containing at least 4 animals reproducible average values were obtained. Accumulation of citrate in some of the tissues from irradiated fluoroacetate-treated rats was markedly depressed soon after a lethal dose of X-ray. As shown in Fig. 1 spleen and thymus were affected to the greatest extent among the tissues examined. A considerable decrease in citrate accumulation was observed when fluoroacetate was given within 20 minutes after irradiation and the animals were sacrificed in 3 hours. Within 12 hours after irradiation the depression of citrate accumulation reached its maximum value and no measurable reversal of the effect was observed throughout the survival time (*ca* 5 days). Some inhibition of

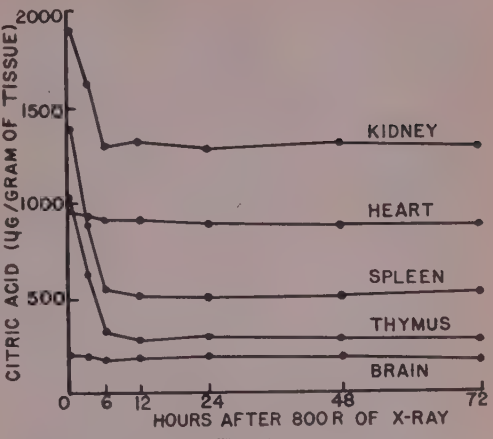

FIG. 1.
Effect of 800 r of X-ray on accumulation of citric acid in tissues of fluoroacetate-treated rats.

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TABLE I. Effect of X-Irradiation on Citric Acid Accumulation in Tissues from Fluoroacetate-Treated Rats.

Tissue	μg citric acid/g fresh tissue	
	Control	48 hr after 800 r
Ileum	736 (680- 816)	147 (94-176)
Pancreas	884 (704-1064)	76 (50-109)
Testes	168 (150- 225)	79 (61-110)
Skeletal muscle	88 (60- 104)	58 (45- 79)
Liver	64 (44- 71)	177 (100-384)

citrate synthesis by kidney was also observed. The absence of an effect by irradiation on citrate synthesis by heart and brain was significant because these tissues are generally considered(15,16) to be especially resistant to injury by ionizing radiations. This finding would seem to eliminate the possibility that incomplete absorption of fluoroacetate after intraperitoneal administration was responsible for the inhibitory effects observed in other tissues.

A survey of the effects of 800 r of X-ray on citrate synthesis in rat tissues other than those shown in Fig. 1 was also conducted. For this experiment rats were sacrificed at 48 hours after 800 r. Non-irradiated controls similarly treated with fluoroacetate were included for comparison and the results are shown in Table I. A marked inhibition of citrate synthesis was observed in ileum, pancreas and testes in 48 hours after 800 r of X-ray while a much less significant decrease occurred in skeletal muscle. Whereas 800 r of X-ray either exerted no effect or inhibited citrate synthesis in all other tissues liver exhibited an increase in citrate content after fluoroacetate treatment. It has previously been shown(11,13) that the liver of normal male rats does not accumulate citrate after fluoroacetate treatment. However, liver is capable of producing large quantities of citrate *in vitro*(11) and it is therefore clear that a mechanism preventing the accumulation of citrate in the liver after fluoroacetate treatment is operative in normal male rats. The fact that the livers of irradiated male rats accumulated large amounts of citrate following fluoroacetate treatment indicates that a marked change in the metabolic pattern of the liver has occurred as a result of irradiation damage.

After the demonstration of the inhibitory effect of 800 r of X-ray on citrate accumulation in rat tissues further experiments were conducted to ascertain the effect of other doses of X-ray on citrate synthesis. For these tests groups each containing 4 rats were given fluoroacetate 21 hours after irradiation and were sacrificed 3 hours later for citrate determinations on spleen, thymus and kidney. After 100 r of X-ray citrate accumulation was inhibited to the extent of 27% in thymus and 21% in spleen whereas 200 r caused 68% inhibition in thymus and 53% inhibition in spleen in 24 hours after irradiation. After higher doses of radiation (400 r to 1200 r) 60% to 85% inhibition of citrate accumulation occurred in spleen and thymus and a definite relationship between dosage of X-ray and amount of inhibition was no longer apparent. Kidney showed no significant decrease in ability to accumulate citrate following fluoroacetate treatment when doses of X-ray below 800 r were given to rats. The results of this experiment showed that the inhibition of citrate accumulation in rat tissues varies with the dose of X-ray and with the known sensitivity(15,16) of the particular tissue to injury by X-irradiation.

A few tests were conducted on tissues from mice and guinea pigs to observe whether the inhibition of citrate synthesis by X-irradiation also occurred in tissues from these species. The fluoroacetate treatment consisted of administration of 2 mg/kg to guinea pigs and 10 mg/kg to mice and the animals were sacrificed 3 hours after fluoroacetate treatment. In 24 hours after administration of 100 r of X-ray to young guinea pigs citrate accumulation decreased to the extent of 43% in spleen and 65% in thymus. The average citrate values for these tissues from normal, fluoroacetate-treated guinea pigs were 344 μg/g for thymus and 448 μg/g for spleen.

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Tests on mouse spleen were done by pooling the spleens from 5 animals. Whereas the spleens of normal mice contained an average of 1272 μg/g the value was depressed to 528 μg of citric acid/g of tissue representing 59% inhibition in 24 hours after 200 r of X-ray. These tests demonstrated that low doses of irradiation produce marked inhibition of citrate synthesis in mouse and guinea pig tissues. Attempts to correlate species susceptibility to irradiation with inhibition of citrate synthesis cannot be made until more detailed studies are conducted on mice and guinea pigs.

Since sublethal doses of irradiation produced marked inhibition of citrate synthesis in spleen and thymus it was possible to study the reversibility of the effect by performing assays at intervals for several days after single doses of X-ray. For this study two series of rats were given 200 r and 400 r of X-ray and groups each containing 4 animals were given fluoroacetate at intervals from 1 to 14 days following irradiation. Selection of the doses of X-ray and the time intervals for the citrate assays was based on the results of preliminary trials which demonstrated reversibility of the inhibition after sublethal doses in contrast to the irreversible inhibition (Fig. 1) after lethal doses. The results of measurements of the rate of reversal of inhibition of citrate synthesis after 200 r and 400 r of X-ray are shown in Fig. 2 in which each value represents an average for 4 rats. A marked inhibition of citrate synthesis occurred in 24 hours after irradiation as may be seen by comparing the normal values in Fig. 1 with the initial values shown in Fig. 2. After 200 r of X-ray reversal of the inhibition of citrate synthesis in spleen and thymus began soon after irradiation and progressed until the activity reached the normal values for these tissues in 14 days after irradiation. After 400 r the initial inhibitory effect at 1 day after X-ray was considerably greater than that observed when 200 r was given. In addition, no appreciable recovery occurred during the first 5 days after irradiation. However, after the initial lag period reversal of the inhibition took place rather rapidly although the values remained somewhat below normal at 14 days as shown in Fig. 2. It should be noted that a small percentage of the rats succumbed to 400 r within 7 days after X-irradiation and animals used at later periods thus necessarily represent individuals which were more resistant to radiation.

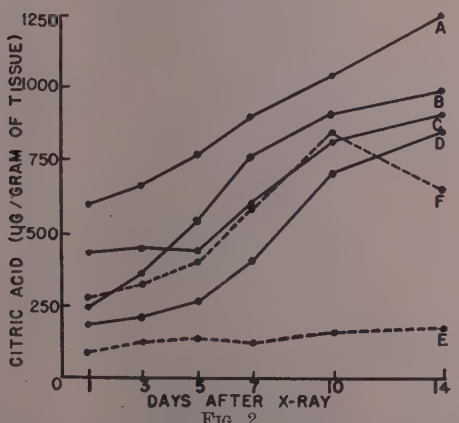

FIG. 2.

Duration of effects of single doses of X-ray on ability of rat tissues to accumulate citric acid after fluoroacetate treatment. A. Spleen, 200 r. B. Thymus, 200 r. C. Spleen, 400 r. D. Thymus, 400 r. E. Liver, 200 r. F. Liver, 400 r.

Liver was included in the experiment shown in Fig. 2 because of the evidence given in Table I that a rise in the citrate content of this tissue occurs after irradiation and fluoroacetate treatment. The results of this experiment are shown in Fig. 2 in which each value represents an average for 4 male rats. After 200 r and fluoroacetate treatment there occurred an increased citrate level in the liver which persisted at values between 100 and 200 μg/g throughout the 14-day period. This effect was much more striking in animals which received 400 r in which the citrate value rose to an average of 830 μg/g of tissue in 10 days after irradiation. Pretreatment with fluoroacetate was necessary to obtain the citrate accumulation in liver of irradiated animals as evidenced by an average value of 64 μg of citrate/g of tissue in 3 days after 400 r when no fluoroacetate was given and 325 μg/g after fluoroacetate was given. Individual variations in the citrate values for liver after 400 r were considerably greater than were observed with other tissues. Thus, we

have obtained citrate values ranging from 400 to 1980 μg/g of tissue at 7 days after 400 r. With this rather wide range of values the average may be expected to vary from one series of animals to another under the conditions employed in this study. However, the ability of the liver of male rats to accumulate citrate after irradiation and fluoroacetate treatment has been a consistent finding throughout our experiments. In tests on normal female rats (180-260 g) we have found that citrate accumulates in the livers of non-irradiated animals after fluoroacetate treatment as evidenced by values ranging from 248 to 1540 μg of citrate/g of liver in 12 animals. Young female rats (55-65 g) resembled normal male rats in that fluoroacetate failed to cause citrate accumulation. These observations suggest that an effect by X-ray on steroid hormones may be responsible for the citrate accumulation observed in the livers of irradiated male rats following fluoroacetate treatment.

Since growth rate is commonly used as an index of radiation damage it was of interest to compare the changes in body weight and the biochemical changes observed in these experiments. Groups each containing 20 male 200-g rats were given 200 r and 400 r of X-ray and body weight changes were recorded for 2 weeks. Fig. 3 shows the per cent change in the initial body weight of irradiated and control animals. Comparison of the data in Fig. 2 and 3 shows that reversal of the inhibition of citrate synthesis in spleen and thymus started immediately before the animals began to gain weight. However, the growth rate remained below normal and the liver effects persisted throughout the 14-day observation period. Thus, elucidation of any possible relationship between body weight changes and the biochemical effects observed in this study must await further studies on the specific nature of the biochemical changes in liver and other tissues after X-irradiation.

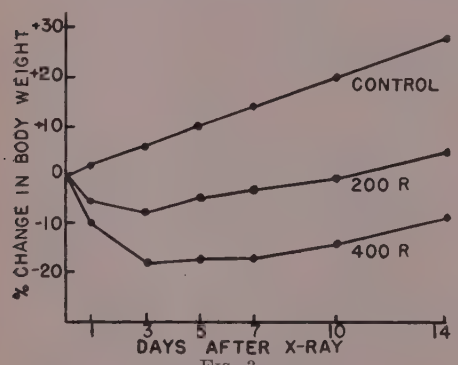

FIG. 3.
Effect of X-ray on growth rate of rats.

Discussion. The results of these experiments indicate that X-irradiation inhibits the accumulation of citric acid in some tissues after treatment of animals with fluoroacetate according to the procedure of Potter(5). The inhibitory effect was greatest in organs such as the spleen, thymus and intestine which are especially susceptible to injury by ionizing radiations(15,16). These observations show that one of the repercussions of X-ray irradiation is the inhibition of one or more reactions within the Krebs cycle or other reactions which ultimately lead to citrate accumulation following injection of fluoroacetate. Potter(5) has pointed out that the specific cause of inhibition of citrate accumulation in fluoroacetate-treated animals by a toxic agent could be far removed from direct interference with a reaction within the Krebs cycle. Whether the effects of X-ray on citrate accumulation are direct or indirect remains to be shown and further studies to test for possible changes in specific enzymes must therefore be made. While inhibition of sulfhydryl enzymes(3,4) might be considered as a possible explanation for the results obtained in this study, experiments in this laboratory (to be published) have shown that lethal doses of radiation cause no appreciable decrease in the activity of succinic dehydrogenase, adenosine triphosphatase and other sulfhydryl enzymes of several tissues *in vivo*. It, therefore, seems worthy of emphasis that sulfhydryl enzymes which are inhibited by ionizing radiations *in vitro* are not necessarily inhibited in the intact animal.

Accumulation of citrate in the livers of fluoroacetate-treated rats after X-ray presented an interesting deviation from normal male rats in which fluoroacetate fails(11,13) to cause citrate accumulation. It is note-

worthy that this effect was prominent after sublethal doses of X-ray indicating that some metabolic function of the liver is markedly altered by X-irradiation. The present biochemical studies have thus revealed metabolic changes in the livers of irradiated animals which are apparently not accompanied by detectable pathological changes(15,16). The observation that normal female rats can accumulate citrate in the liver after fluoroacetate treatment in contrast to normal male rats suggests that this effect is regulated by the sex hormones. Citrate accumulation in the livers of X-irradiated male rats thus suggests that some of the acute effects as well as delayed effects may result from alteration of steroid hormones by X-ray.

Summary. The technic of Potter(5) for measuring the effects of toxic agents on citrate accumulation *in vivo* was applied to studies on X-irradiation. Lethal doses of X-ray (800 r) markedly inhibited citrate accumulation in spleen, thymus, ileum, pancreas and testes of fluoroacetate-treated rats but exerted no significant effect on brain and heart. Sublethal doses of X-ray markedly inhibited citrate accumulation in spleen and thymus. The extent of inhibition was dependent upon the dose of X-ray and was reversible after sublethal doses and irreversible when lethal doses were given. Accumulation of citrate occurred in the livers of irradiated rats following fluoroacetate treatment in contrast to the inability of livers of normal male rats to accumulate citrate following fluoroacetate treatment.

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Colorimetric Measurement of Serum Cholinesterase. (18512)

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Immense quantities of phosphorus-containing anticholinesterase agents are currently in use in this country as agricultural insecticides. The high toxicity of these materials to man has resulted in a number of cases of poisoning (1) including some fatalities among people inadvertently exposed during the manufacture and use of these compounds. Since some of these agents, especially parathion and octamethyl pyrophosphoramide (OMPA, Pestox III), may exert a cumulative effect(2-4), serious poisoning can occur in individuals who have been exposed to relatively small amounts of these materials repeatedly. Periodic measurements of the cholinesterase levels of the blood of workers handling the anticholinesterase agents provide the only known means of detecting exposure before the advent of symptoms. The Warburg manometric method is entirely suitable for these measurements, but the necessary special equipment and personnel trained in its use are often not available under the conditions of practical usage of these agents. A need has, therefore, arisen for a simple, accurate, and economical method for measuring the cholinesterase activity of the blood of persons exposed to anticholinesterase agents. In efforts to devise a suitable procedure we have found that the colorimetric esterase method of Gomori(5) can be adapted as an assay procedure for the pseudocholinesterase of serum. This procedure is not applicable to measurements of the true

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cholinesterase of red cells, but since inhibition of serum cholinesterase generally occurs sooner than inhibition of the enzyme activity of other tissues after exposure to anticholinesterase agents, values for serum may be used as an indication of exposure.

The present communication describes the colorimetric measurement of serum pseudocholinesterase and to illustrate the reliability of this procedure a comparison of values obtained by this method and the manometric technic is presented. The applicability of the assay is shown by data obtained on serum of patients under treatment for myasthenia gravis with octamethyl pyrophosphoramide.

Methods. Manometric assays were made according to the method of DuBois and Mangun(6). The colorimetric determinations of cholinesterase activity were made using the esterase method of Gomori(5) with minor modifications. The procedure as employed in our studies was carried out as follows:

Preparation of a standard curve. For this cholinesterase method phenyl benzoate is employed as the substrate and the amount of phenol liberated through hydrolysis by pseudocholinesterase is measured. A stock aqueous solution of phenol (reagent grade) containing 100 μg/ml was prepared. For the standard curve amounts from 2.5 to 25 μg of phenol were diluted to 6 ml with water in colorimeter test tubes. To each of the tubes 4 ml of a saturated borax solution was then added. The borax solution was prepared by adding 35 g of powdered $Na_2B_4O_7$ $10H_2O$ to 1 liter of 15% ethanol and allowing the mixture to dissolve over a 24-hour period. To each tube 0.5 ml of diazo reagent (prepared by dissolving 0.25 g of DuPont Naphthanil Diazo Red B salt in 100 ml of ice-cold water and filtering) was added. The samples were mixed and allowed to stand 10 minutes and were then read against a reagent blank in a Klett-Summerson photoelectric colorimeter with a No. 50 blue-green filter. A straight line relationship was obtained by plotting phenol concentrations against Klett readings.

Serum cholinesterase measurements. For colorimetric measurements of cholinesterase activity, 1 ml of serum was diluted to 100 ml with water and 1 ml of diluted serum (0.01 ml of serum) was placed in a colorimeter tube. Five ml of a buffered phenyl benzoate solution was then added. The buffer was prepared by dissolving 5.35 g of $Na_2HPO_4 \cdot 12H_2O$ and 7 g of $NaH_2PO_4 \cdot H_2O$ in 1 liter of water. Into 500 ml of this buffer 1 ml of stock substrate solution (2 g of phenyl benzoate in 100 ml of methanol) was added. After addition of the substrate to the serum samples they were incubated at 37°C for 1 hour. The samples were then placed in ice-cold water and the borax and dye reagent was added as described above. Blank samples were included in which the serum was added after the incubation at 37°C. The micrograms of phenol liberated per 0.01 ml of serum represented the serum cholinesterase activity after deducting the small blank values. The borax solution and the phosphate buffer will keep indefinitely. However, the buffered substrate and the dye reagent must be kept in a refrigerator and will then only keep about 2 weeks.

Results. To test the applicability of the colorimetric method for serum cholinesterase, determinations were made on the serum of 40 normal persons. Simultaneous manometric measurements were made on the same samples. By the colorimetric method the average cholinesterase activity expressed in μg of phenol liberated by 0.01 ml of serum/hour was 23 with individual values varying from 16 to 30. The average value by the manometric method was 36 expressed as μl of CO_2 liberated/5 min/0.1 ml of serum and individual values ranged from 27 to 46. The variation of in-

TABLE I. Comparison of Colorimetric and Manometric Methods for Measuring Inhibition of Serum Cholinesterase *in Vivo*.*

% of control activity			
Manometric method	Colorimetric method	Total mg of OMPA	Avg No. days of treatment
100	100	0	0
75	74	12.6	1.5
55	48	36.8	3.5
35	31	70.2	6.5

* Avg values for 5 patients under treatment with octamethyl pyrophosphoramide (OMPA) 8 to 12 mg/day.

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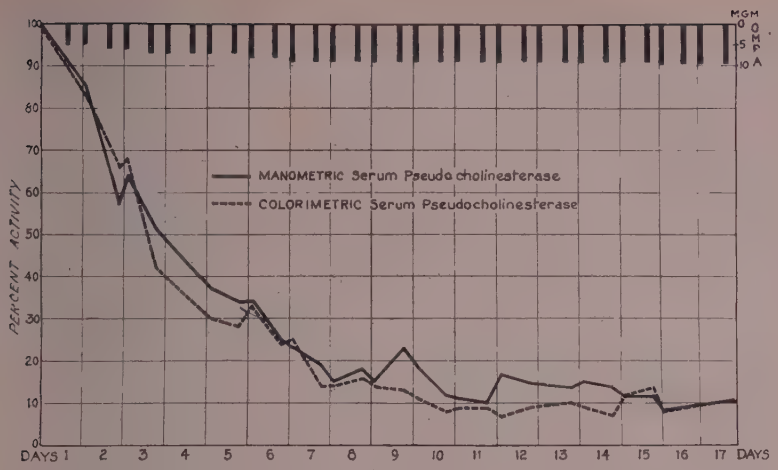

FIG. 1.

Depression of pseudocholinesterase by OMPA as determined manometrically and colorimetrically.

dividual samples from the average was similar with both methods. Thus, high or low cholinesterase activity among different individuals could be detected by either method.

In vitro experiments with neostigmine and di-isopropyl fluorophosphate (DFP) demonstrated that the inhibitory effect of the phenylcarbamic acid esters, as well as the organic phosphates on serum cholinesterase, can be measured by the colorimetric method. Thus, a final concentration of 7.5×10^{-7} M neostigmine produced 50% inhibition of serum cholinesterase by the colorimetric method, whereas 6×10^{-7} M neostigmine produced 50% inhibition using the manometric technic when the inhibitor was mixed with the serum before addition of the substrate. The final concentrations of DFP which produced 50% inhibition of serum cholinesterase were 6.3×10^{-8} M and 4.5×10^{-8} M by the manometric and colorimetric methods respectively.

The practical application of the colorimetric determination of cholinesterase activity was studied using serum from 5 patients with myasthenia gravis who were under treatment(4) with octamethyl pyrophosphoramide (OMPA). After measurements of the normal serum cholinesterase activity were made on each patient, oral administration of OMPA was begun and continued at an average dose of 8 to 12 mg/day in 2 divided doses. Serum cholinesterase was usually determined twice each day by the colorimetric and manometric methods. The data in Table I give a comparison of the average % depression of enzyme activity in the 5 patients as measured by both methods. It may be seen that there is a close correlation between the inhibition observed by the two methods.

A more detailed comparison of the daily depression of cholinesterase as measured by the colorimetric and manometric methods is shown in Fig. 1 which shows the decrease in enzyme activity following daily administration of OMPA to a patient with myasthenia gravis. Throughout the observation period there was good correlation between the results obtained by the two methods which indicates that the colorimetric procedure may be used as a reliable index of exposure to anticholinesterase agents.

Summary. The colorimetric esterase method of Gomori(5) was applied to studies on the cholinesterase activity of serum. The method was found to be applicable to studies on the action of anticholinesterase agents *in vitro*. It is also suitable for detecting exposure of humans to organic phosphorus-containing insecticides and for following serum cholinesterase levels during therapy with anticholinesterase drugs.

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Effect of Nitrogen Mustard on Serum Complement *in vitro* and in Patients with Neoplastic Disease. (18513)

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The object of the present study was threefold. First, to determine whether serum complement titers were normal in patients with neoplastic disease, especially of the lymphomatous types.† This seemed of interest since complement titers have been reported to be abnormal in certain infectious diseases(5,6). Second, to ascertain whether nitrogen mustard inactivates human complement *in vitro,* as it does guinea pig complement(1). Third, to determine whether the clinical administration of nitrogen mustard causes depression of complement titer. Because of clinical availability, this third part of the investigation was carried out with patients who received x-radiation in addition to nitrogen mustard, and hence the effect of x-radiation on complement titer has also been studied.

Methods. All sera were separated from the clot, frozen at –76°C within 2 hours of venipuncture, and were kept in deep freeze until titrated. Complement was measured by the technic of Kent, Bukantz, and Rein(4) in terms of the 50% hemolysis unit. Results are recorded as the fraction of a milliliter containing one unit of complement.

Complement titers in patients with neoplastic disease. The serum complement titers of 42 patients with neoplastic diseases, mostly lymphomas and leukemias, are presented in Table I, together with complement titers of 12 healthy adult controls. There was a greater range of variation among the complement titers of the patients than in the controls, but there was no significant difference between the mean titers of the several groups. Several sera were retitered after an interval of several days (at –76°C) with close reproduction of results.

TABLE I. Complement Titers of Patients with Neoplastic Diseases.

Diagnosis	No. of cases	50% hemolysis unit (ml) Range	Mean	S.D.
Ac. leukemia	6	.0015-.0065	.0030	.0012
Chr. myel. leukemia	7	.0023-.0072	.0047	.0017
Chr. lymph. leukemia	5	.0023-.0065	.0040	.0013
Lymphosarcoma	7	.0021-.0045	.0032	.0007
Hodgkin's disease	7	.0021-.0045	.0035	.0009
Bronchogenic Ca.	6	.0034-.0075	.0055	.0013
Breast Ca.	2	.0049-.0050	.0050	—
Nephroma	1	—	.0048	—
Osteogenic Sa.	1	—	.0058	—
All neoplasms	42	.0015-.0075	.0041	.0009
Normals	12	.0027-.0057	.0041	.0009

Effect of nitrogen mustard on serum complement in vitro. Freshly drawn serum from a normal male was divided into 6 portions and diluted with equal volumes of 0.85% saline or various concentrations of methyl-bis-beta-chloroethyl-amine hydrochloride dissolved in 0.85% saline. One sample of saline-diluted serum was then placed in a refrigerator at 3°C for 2 hours, while another saline control and all of the nitrogen mustard treated sera were kept at 37°C for two hours. Complement was then titered. This experiment was performed twice. Data are recorded in Table III. The nitrogen mustard in a concentration of 1.0 or 10.0 mg per ml of serum completely inactivated complement, but no significant change in complement titer was produced by the lower concentrations, as compared to the control subjected to the same conditions of incubation. The control incubated for 2 hours at 37°C showed a 25% to 50% loss of complement activity as compared with the control maintained at 3°C.

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† This phase of the study was done in cooperation with Dr. Anna Dean Dulaney formerly of this institute.

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TABLE II. Serial Complement Titers in Cancer Patients Before and After Treatment with 0.4 mg/kg Nitrogen Mustard Plus Radiotherapy, and in Untreated Controls.

			50% hemolytic unit (ml)						
				Days post Rx					
Age	Sex	Dx	pre Rx	1	2	3	4	5	11
73	M	Broncho	.0075	.0067	.0088				
62	F	Nephroma	.0048	.0054					
56	M	Broncho	.0055	.0055	.0058				
60	M	"	.0064		.0063	.0042			
31	F	Breast	.0049	.0057	.0048	.0056	.0066		
65	M	Broncho	.0050	.0050					
52	M	"	.0049		.0048			.0046	
60	M	"	.0034	.0033			.0035		
34	M	Osteo.	.0058	.0062		.0062			.0056
33	F	Untreated breast	.0050	.0053	.0057	.0054	.0046	.0051	
30	M	Untreated normal	.0079	.0081	.0078	.0058		.0059	

TABLE III. Effect of Nitrogen Mustard on Human Complement Activity *in Vitro*.

N mustard conc. mg/ml of serum	2 hr incubation, °C	ml of serum containing 1 unit complement	
		Exp. 1	Exp. 2
0	3	.0081	.0067
0	37	.015	.0085
.01	37	.012	.0090
.10	37	.013	.0089
1.00	37	>.100	>.1000
10.00	37	>.100	>.1000

Serial complement titers after nitrogen mustard and x-ray therapy. Serial specimens of blood were obtained from 9 patients before, and for one to 11 days after, injection of nitrogen mustard. Each patient received a single dose of 0.4 mg/kg of methyl-bis-beta-chloroethylamine hydrochloride, given intravenously while circulation of both legs and one arm was completely occluded by tourniquets. Tourniquets were left in place for 5 to 10 minutes after the injection in order to concentrate the nitrogen mustard in the trunk during its short period of cytotoxic activity(2). The maximum theoretical concentration of nitrogen mustard that might occur by use of this method is approximately 0.01 mg/cc of serum (assuming a plasma volume of 5% of body weight, complete intravascular mixing, 50% of blood volume isolated by tourniquets, and no immediate intracellular or extra-vascular diffusion). Immediately after the nitrogen mustard injection, intensive fractionated-dose radio-therapy was given to the diseased areas from a million volt machine, and was repeated daily during or beyond the period of the complement studies.‡ Most of the patients had x-ray directed to the thoracic area. Dosage was in the range of 2000 r tumor dose in a period of 7 to 14 days. The patients' diagnoses are indicated in Table II. Two untreated controls, one normal and one cancer patient, were included in the study in order to determine the normal fluctuation in day to day complement titers. It is apparent from the data (Table II) that no significant variations in complement titer occurred. Daily fluctuations in the controls are as great as the changes in the treated patients.

Because it seemed possible that the nitrogen mustard (or x-ray) treatment might have caused an immediate drop in complement which was obscured in the above studies by rapid regeneration of complement in the first 24 hours after treatment, blood was drawn

‡ The clinical efficacy of this combined method of therapy for inoperable neoplasms is being evaluated at Memorial Hospital by Drs. Ralph Phillips and D. A. Karnofsky, and is based on the additive effects of nitrogen mustard and x-rays in certain sequences in animals(3).

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from one patient immediately before injection of the nitrogen mustard, 5 minutes after injection but before tourniquets were released, 2 minutes after tourniquets were released, and 3 hours after treatment was completed. No significant change occurred. The titers, in the order in which the specimens were taken, were 0.0058 ml, 0.0061 ml, 0.0060 ml, and 0.0062 ml per 50% hemolysis unit.

Discussion. This study indicates that in the neoplastic diseases studied there is no consistent deviation of complement activity from normal, although greater variability may occur than in normals. In a series of patients with various infectious diseases, Ecker *et al.*(6) had similar findings. These workers, however, found frequent high complement titers in streptococcus and pneumococcus infections, and frequent low titers in meningococcus meningitis, which differences they felt to be significant. Dulaney(5) has reported frequent low complement titers in malaria. Ecker *et al.*(6) reported a possible relationship of complement to severity of illness and serum protein concentration, but no relation to fever or leukocytosis. In the present work serum proteins were not studied, but there was no apparent relation between complement titer and extensiveness of disease, fever, degree of debility, leukocyte count, or transfusions.

The results of the present *in vitro* experiments with nitrogen mustard and human complement are in general agreement with the results reported with guinea pig complement by Watkins and Wormall(1). The absence of any change in complement titer following clinical use of nitrogen mustard is what would be expected from a consideration of the *in vitro* studies, since the concentrations of mustard attained in patients are far below those which destroyed complement *in vitro*.

Conclusions. The mean serum complement titers of patients with various neoplastic diseases did not differ significantly from that for normal adults. The range of complement values was greater among cancer patients than among normals, but the erratic titers did not appear to be related to clinical status. No depression of human complement titer was observed following the clinical use of nitrogen mustard and x-ray in maximal dosage. Human complement is inactivated by high concentrations of nitrogen mustard *in vitro*, but not by concentrations in the range which is achieved in clinical usage.

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Influence of Vitamin B_{12} and Liver Extract on Nitrogen Balance of Normal and Hyperthyroid Rats.* (18514)

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The retardation of growth observed upon feeding thyroid hormone can be prevented by simultaneous administration of liver preparations in growing mice(1), chicks(2), rats(3-7), and rabbits(8). Similar effects have been obtained in rats with vitamin B_{12} (8,9), and with various liver preparations high in antipernicious anemia activity(8). Other

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† Dept. of Medicine.

‡ Depts. of Physiology and of Medicine.

§ Dept. of Biochemistry.

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B vitamins which prevent weight loss in the hyperthyroid mature rat(10) are ineffective in this respect in the immature hyperthyroid rat (3).

Prevention of weight loss and continued growth of the hyperthyroid animal by administration of liver preparations or vitamin B_{12} has been studied in animals fed *ad libitum*. It is believed that increased food intake is in part responsible for the beneficial results of treatment. However, it has been suggested that increased utilization of food may play a role in the prevention of weight loss and/or continued growth of hyperthyroid animals treated with these preparations(4). It was therefore considered of interest to study the influence of liver preparations and of vit. B_{12} on the nitrogen balance of normal and hyperthyroid rats on a constant food intake.

Material and methods. Male rats, descendants of the Wistar strain (Albino Farms, Red Bank, N. J.) weighing between 190 and 250 g, were used. All animals were tube fed the "mixed diet" described by Ingle *et al.* (11)‖ twice daily. The animals were gradually adapted to tube feeding, and were then fed a constant amount during the control and the experimental period, the amount selected for each animal being sufficient to maintain weight, or to permit a slight weight gain during the control period.

The animals were kept in a constant temperature room in wire screened, individual metabolism cages. Urine specimens were collected at 24 hour intervals in collecting bottles containing 1 g of citric acid, and were analyzed daily by a micro-Kjeldahl method. The urinary N-excretion and the weight of each animal was recorded daily for a control period of 9 days. This was followed by an experimental period of equal length, during which various preparations were injected subcutaneously. Groups 1 and 2 represent normal rats, injected during the treatment period with vit B_{12} and liver extract respectively. Group 3 was made hyperthyroid by daily injection of 150 γ d-l thyroxin. Groups 4 and 5 were made hyperthyroid in the same way as group 3, but received vit B_{12} and liver extract respectively. All injections were given at the same time each day.

Results. Results are recorded in Table I, and in Fig. 1. Exp. 1 and 2 show that neither vit B_{12} nor liver extract influenced the weight or nitrogen excretion of the adult rat on constant food intake.

The results of Exp. 3, 4 and 5 demonstrate that the protein catabolic effect of thyroxine was not abolished by simultaneous treatment with either vit B_{12} or liver extract; in spite of this treatment the urinary N-excretion increased and the animals lost weight. In Fig. 1 the percentage weight loss and nitrogen loss in rats injected with thyroxine alone (Exp. 3) is compared with that of the thyroxine-treated animals receiving vit B_{12} or liver extract (Exp. 4 and 5). The thyroxine-induced nitrogen loss was significantly decreased in the animals treated with vit B_{12}. The N-sparing effect of liver extract, with the amounts employed, was slight, and statistically not significant. The weight loss, however, did not differ significantly in the 3 groups.

Discussion. Vit B_{12} and liver extract failed to induce weight gain or nitrogen retention in the adult rat fed constant amounts of an adequate diet. This indicates that these "growth factors," given in excess, do not exert

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‖ Diet composition:

Constituent	g
Dried yeast	100
Salt mixture (Osborne & Mendel)	40
Wheat germ oil	10
Cod liver oil	10
Cellu flour	120
Mazola oil	175
Whole milk powder	600

This diet contains approximately 16% protein.

TABLE I. Effect of Vit. B_{12} and of Liver Extract on N Excretion of Normal and Thyroxin Treated Rats Force Fed Constant Rations. Nitrogen excretion/24 hr.

Exp.	Treatment	No.	Control period*	Treatment period*	Diff.	"p" of diff.	Wt change g/9 days
1	Vit. B_{12}, 3 γ twice daily	1	221	231	+10	<.3	+ 0.4
		2	231	215	—16	<.2	+ 2.0
		3	196	201	+ 5	<.4	+ 1.5
		4	232	225	— 7	<.5	+ 0.5
		5	238	234	— 4	<.5	+ 1.5
		6	254	242	—12	<.2	+ 3.0
		7	211	202	— 9	<.3	+ 5.5
2	Liver extr., 0.4 U (0.2 ml)	1	195	197	+ 2	<.9	+ 4
		2	196	204	+ 8	<.1	+ 6
		3	200	193	— 7	<.9	+11
		4	185	189	+ 4	<.6	+ 7
		5	197	196	— 1	<.9	+ 4
3	Thyroxin, 150 γ daily	1	174	246	+72	<.01	—23
		2	174	241	+67	<.01	—30
		3	166	227	+61	<.01	—17
		4	193	304	+111	<.01	—54
		5	158	233	+75	<.01	—30
		6	166	218	+52	<.01	—28
		7	140	217	+77	<.01	—23
4	Thyroxin, 150 γ daily Vit. B_{12}, 3 γ twice daily	1	227	283	+56	<.01	—23
		2	254	296	+42	<.01	—35
		3	234	311	+77	<.01	—37
		4	239	259	+20	<.05	—21
		5	229	285	+56	<.01	—33
		6	234	273	+39	<.01	—29
		7	230	278	+48	<.01	—30
		8	220	268	+48	<.01	—24
5	Thyroxin, 150 γ daily Liver extr., 0.4 U (0.2 ml) daily	1	167	216	+49	<.01	—18
		2	183	260	+77	<.01	—28
		3	207	250	+43	<.01	—23
		4	174	263	+89	<.01	—15

* Mean of 9 days.

an anabolic effect. This is in agreement with observations on growing animals(12) and on humans(13), which suggest that liver factor and vit B_{12} accelerate growth mainly by increasing appetite and food intake.

Thyroxine-induced weight loss is not prevented by either vit B_{12} or liver extract in adult rats fed a constant ration. This finding is in agreement with observations on growing animals that the prevention of weight loss, or the continued growth of the thyroid-treated growing animal is due largely to increased food intake(4) and not to neutralization of, or interference with the calorigenic and other metabolic effects of excess thyroid hormone (4). The importance of the caloric intake is further suggested by the observation that high fat diets also protect growing rats against thyroid-induced weight loss(14).

The fact that the nitrogen loss induced by thyroxine, while not abolished, was significantly decreased by vit B_{12} in the doses employed, suggests that this factor has an action on intermediate metabolism in addition to the influence of food intake. Since, in the force fed rat, on constant intake, the thyroxine-induced weight loss is unchanged, but the nitrogen loss is decreased by treatment with vit B_{12}, it must be concluded that through the action of this factor protein is spared at the expense of other nutrients. This could conceivably be related to the "improved efficiency of food utilization" suggested by Ershoff as a second mechanism in addition to the increased food intake in the *ad libitum* fed animal(4).

Summary. Vit B_{12} or liver extract ad-

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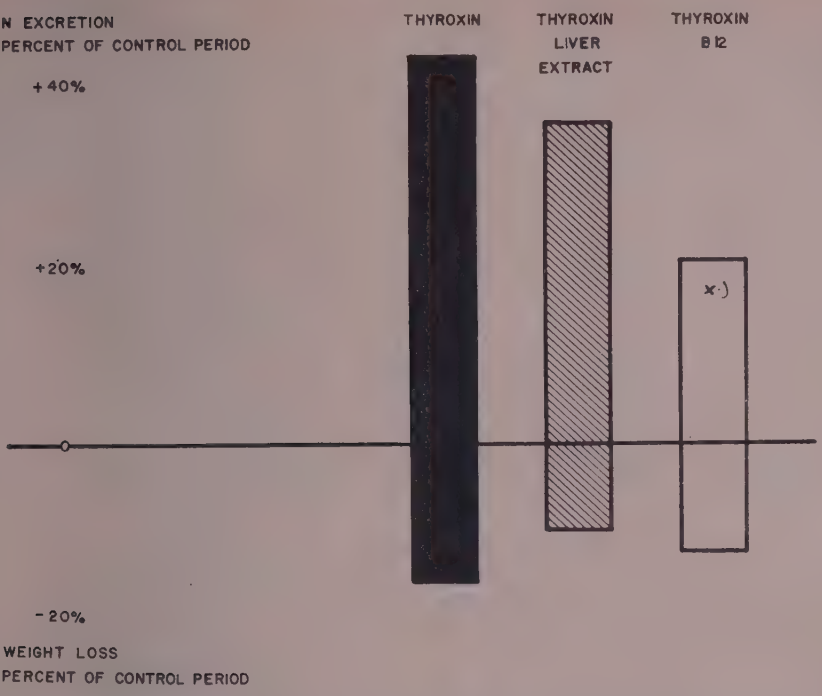

FIG. 1.

N excretion and weight loss. Upper bars indicate increase of N excretion in percent of control period. Lower bars indicate weight loss in percent of weight during control period. x Difference against column one significant $p<0.01$.

ministered to force-fed rats on constant food intake induced neither weight gain nor N retention. When vit B_{12} was administered to force-fed rats on constant food intake, made hyperthyroid by injection of thyroxine, the weight loss was identical with that of hyperthyroid animals not receiving vit B_{12}. The N loss resulting from the catabolic action of thyroxine, however, was significantly smaller in thyroxine-treated rats receiving B_{12} than in the thyroxine-treated controls. Liver extract had a similar action, but the values obtained were statistically not significant. These results suggest that in hyperthyroid rats vit B_{12} spares protein at the expense of other body constituents.

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Effect of Aureomycin and Chloramphenicol on Two Recently Isolated Strains of *Listeria monocytogenes*. (18515)

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A survey of the literature revealed no reports on the effects of aureomycin and chloramphenicol on infections with *Listeria monocytogenes*. Two strains of this organism were recently isolated and found to be virulent for mice. In the present study, the effects of chloramphenicol and aureomycin on these strains were compared *in vitro* and *in vivo*.

TABLE I. Comparative Survival Rates of Mice Infected Experimentally with *L. monocytogenes* and Treated with Aureomycin and Chloramphenicol.

Antibiotic	Dosage†	No. of mice	Strain of *L. monocyt.*	No. surviving at indicated days after infection					% surviving
				5	10	15	20	25	
Aureomycin	10	25	447	25	23	22	20	20	80
"	25	25	447	25	25	24	24	24	96
"*	50	25	447	25	25	25	Not held		100
Chloramphenicol	25	50	447	21	15	15	14	11	22
"	50	50	447	30	20	19	19	15	30
"	100	50	447	29	17	12	12	9	18
Untreated	—	50	447	8	4	3	2	1	2
Aureomycin	25	50	2597	49	49	49	44	43	86
"	50	50	2597	50	50	50	47	45	90
Chloramphenicol	25	50	2597	15	12	10	9	9	18
"	50	49	2597	27	23	20	18	17	35
"	100	50	2597	24	11	10	10	7	14
Untreated	—	50	2597	12	8	8	8	8	16

* Aureomycin 50 mg/kg dose given 4 hr after culture.
† Dosage in mg/kg body wt administered daily for 5 days.

Materials and methods. One strain (2597) of *L. monocytogenes* used in this study was isolated from human spinal fluid; the other (447) was isolated from apparently healthy ferrets(1). Both strains conformed to the morphological and biochemical pattern of the species(2) and both produced monocytosis in rabbits. A third strain (9525), obtained from the American Type Culture Collection, was not lethal for mice in a 10^{-2} dilution, hence was included only in the *in vitro* determinations. The effectiveness of both antibiotics against the organisms *in vitro* was determined by a modified turbidimetric procedure(3). The end point is designated as that concentration of antibiotic which inhibits the growth of the test organism by 50% as determined graphically by plotting the per cent growth $\left(\frac{\text{Optical density test culture}}{\text{Optical density control}} \times 100\right)$ against the logarithm of the antibiotic concentration. A Bagg strain of Swiss mice (11-15 g) was employed for all animal studies. Mice of the maximum and minimum weights were represented in each test group.

Preliminary drug tolerance studies, using a wide range of subcutaneous dosage of aureomycin and chloramphenicol, showed that 25, 50 and 100 mg of aureomycin or chloramphenicol injected daily for 5 days were tolerated, but not 250 mg of aureomycin in 3 daily injections or 500 mg in a single injection. Mice were inoculated subcutaneously with cultures of *L. monocytogenes* grown in dextrose veal infusion broth until 0.145 optical density was obtained at a wavelength of 650 millimicrons. Inocula consisted of 0.5 ml of a 10^{-4} dilution of the standardized cultures. To determine the effect of aureomycin or chloramphenicol on Listeria infection in mice, injections of each drug were given 24 hours after infection and continued for 5 consecutive days thereafter (except in the case of the 50 mg/kg dose of aureomycin, Table I). Aureomycin was administered in 10, 25 or 50 mg/kg daily doses and chloramphenicol in dosages of 25, 50 or 100 mg/kg per day, in 0.5 ml volumes. The chloramphenicol solutions were stored at 4°C, whereas the extremely unstable aureomycin solutions were either freshly prepared daily from the dry state or stored at –70°C.

Results and discussion. The *in vitro* sensitivities for each of the 3 strains tested were identical. Thus, the 50% end points were

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found in tubes containing 0.833 μg/ml of chloramphenicol and 0.050 μg/ml of aureomycin, indicating that *L. monocytogenes* was considerably more susceptible *in vitro* to aureomycin than to chloramphenicol.

The survival rates of animals inoculated with strains 447 and 2597 are shown in Table I. Thus, a significantly greater number (80-100%) survived after being treated with aureomycin as compared with those receiving chloramphenicol (18 to 35%). Only 2% of the untreated controls survived.

During the course of the study it was noted that mice, both treated and untreated, which died more than 10 days after being infected, often exhibited a marked and progressive paralysis. The first apparent symptom was a dragging of one hind leg, followed by partial immobilization of both lower quarters and eventual paralysis of the entire body except for the head. Three of these paralyzed mice were sacrificed and cultures taken from the brain and heart. In 2 instances *L. monocytogenes* was recovered, once from both brain and heart and once from the brain only.

Under the conditions of the experiments reported, five consecutive daily doses of 50 mg of aureomycin per kg of mouse body weight are necessary to obtain 90 to 100% survival of mice infected with *L. monocytogenes*. With the same dosage of chloramphenicol, however, only 30 to 35% of the mice survived. Treatment of the mice with 100 mg/kg of chloramphenicol did not increase the percentage of survivors.

Summary. 1. Aureomycin is more effective than chloramphenicol in inhibiting growth of three strains of *Listeria monocytogenes in vitro*. 2. More mice survived infection with *L. monocytogenes* when treated with aureomycin than with chloramphenicol.

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Succinate Stimulation of Normal and Tumor Tissue Slice Metabolism Measured by Reduction of Tetrazolium Chloride.* (18516)

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Roskelley *et al.*(1) reported that the addition of succinate or p-phenylenediamine to slices of normal tissues resulted in a marked increase in oxygen uptake. In contrast to this, only a minimal stimulation was observed when different neoplastic tissues were studied. In an extension of this type of investigation, Rosenthal and Drabkin(2) found that some normal tissues also tended to yield a relatively low percentage stimulation by these 2 substrates. At the same time, they confirmed the finding of a low response in diverse tumor tissues. By the clever device of studying the response to the substrate in the presence of added cytochrome c Greenstein was able to show that when tissues change from the normal to the malignant state, cytochrome c is diminished to a much greater extent than is cytochrome oxidase. In short, the limiting factor in the substrate stimulation in the experiments discussed appears to be the components of the cytochrome system(3,4). It has recently been shown that tetrazolium salts may be used to measure dehydrogenase activity of tissues both in the absence and presence of substrates(5-7). While the exact site of

*Aided by grants from the Damon Runyon Memorial Fund for Cancer Research, Inc., to the New York Medical College and the Leukemia Research Foundation, Inc.

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participation of the tetrazolium in the respiratory chain of enzymes is not precisely defined as yet, it would appear that its reduction reflects the participation of dehydrogenase and/or flavoprotein activity(8).

In the course of studies which will be reported elsewhere, we had found that the intensity of the *in vitro* endogenous oxygen uptake by slices of different tissues as measured by conventional manometric technics is paralleled by the intensity of their reduction of tetrazolium chloride. However, under varied experimental conditions the two procedures may be shown to reflect the activity of different components of the respiratory chain of enzymes. It, therefore, appeared worthwhile to test the effect of succinate on the reduction of tetrazolium salts by various normal and tumor tissues and to compare these findings with the previous reports of oxidative stimulation by succinate. The results of the present investigation would indicate that normal and tumor tissue slices may show an identical percentage increase in metabolism upon the addition of succinate. In addition, the presence of a malignant tumor was not shown to produce any change in the endogenous or succinate stimulated metabolism in respect to tetrazolium reduction by kidney, liver or spleen slices of mice as compared with the values found in control animals of the same strain.

Materials and methods. The human tissues consisted of operative specimens obtained within 30 minutes of removal, while the mouse tissues were obtained by rapid removal from animals killed by crushing of the cervical cord. The mice used were of the CFW strain (Carworth Farms, New York) with and without spontaneous breast carcinomas, as well as C57 black mice with and without implanted sarcoma 180. The transplantable sarcoma 180 was kindly furnished by Dr. K. Sugiura of the Sloan-Kettering Institute. The tumor was implanted into adult female C57 black mice and allowed to grow to the size of at least 1 cm in diameter before the animals were sacrificed for analysis. The CFW mice were all females weighing more than 22 g and only the grossly non-necrotic portions of the spontaneous breast tumors were taken for analysis. The tissues to be studied were sliced free hand with a razor to a thickness of approximately 1 mm. In the case of the normal colon and stomach, the mucosa was carefully dissected free and the sheet of tissue then cut into segments approximately 1 cm square. The measurement of the metabolism was performed according to the technic indicated in a previous publication(6). In essence it consists of the following steps: a. The tissue slices are incubated with continuous gentle agitation at 37°C, pH 7.2 in individual wide mouthed test tubes. The incubation solutions consisted of 3 ml of a phosphate buffered 1% solution of 2, 3, 5-triphenyl tetrazolium chloride (TTC) in distilled water plus 1 ml of 0.9% saline for the measurement of the endogenous metabolism, or 1 ml of sodium succinate solution, equivalent to 2×10^{-4} M of succinate. b. After one hour's incubation the solution is decanted and the reduced TTC, which is red in color, is extracted by repeated washings with acetone. c. The acetone washings are added to a colorimeter tube, diluted to the 5 ml mark with acetone and readings taken of the light transmission, using the Klett-Summerson photoelectric colorimeter with the No. 54 green filter. d. The tissues are dried, weighed on an analytical balance and the intensity of color produced per mg of acetone dried tissue per hour is calculated. e. This value is converted to micrograms of TTC reduced per mg of acetone dried tissue per hour by the use of a standard curve obtained by the chemical reduction of known amounts of TTC. It should be mentioned that each determination was run in duplicate.

Results. The results of our studies on a wide variety of normal and tumor tissue types are indicated in Table I. It is readily apparent that the percentage increase in TTC

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7. Rutenberg, A. M., Gofstein, R., and Seligman, A. M., *Cancer Research,* 1950, v10, 113.

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TABLE I. Reduction of TTC by Normal and Tumor Tissues with and without Added Succinate.

Species	Tissue	No. cases	μg TTC reduced/mg Ro.	μg TTC reduced/mg Rs.	% succinate stimulation
Human	Stomach mucosa	11	1.0	1.9	90
	Carcinoma	2	.2	.8	300
	Colon mucosa	9	.8	1.7	110
	Carcinoma	8	.5	1.5	200
	Breast, control	4	1.1	2.1	91
	Carcinoma	26	.6	1.6	160
	Lymph node	1	.3	.9	200
	Lymphosarcoma	2	.3	1.7	460
	Lung carcinoma	1	1.2	2.4	100
	Ovary carcinoma	1	.4	.8	100
	Metastatic ca., primary site unknown				
	Epidermoid	1	.6	1.7	180
	Adenocarcinoma	1	.3	.6	100
	Anaplastic	1	1.3	2.0	54
	Teratoma, benign, embryonic	1	1.0	2.3	130
	Prostate, hypertrophic	1	.2	.6	200
	Ileum, mucosa	2	1.8	3.0	66
	Thyroid, control	6	.5	1.0	100
	Ewing's tumor	1	.5	1.6	220
Mouse	Breast, ca., spontaneous	30	1.2	3.0	150
	Sarcoma 180	1	.4	2.3	470
CFW	Liver, control	33	2.3	7.3	218
	Tumor mice*	52	2.4	7.2	200
	Kidney, control	47	4.3	9.8	128
	Tumor mice	67	4.0	9.5	137
	Spleen, control	24	.7	1.7	140
	Tumor mice	24	.7	2.1	200
C57 black	Liver, control	21	2.6	7.3	180
	Tumor mice†	4	2.3	6.2	170
	Kidney, control	22	4.2	10.2	143
	Tumor mice	6	3.1	8.6	178

* Spontaneous breast carcinoma.
† Transplanted sarcoma 180.

reduction caused by the addition of succinate is not constant in the different tumor types, nor is the range of values more limited than that found in the comparable control tissues. It is especially interesting to compare the succinate stimulation obtained in the human tissues from the stomach, colon, breast and lymph node since in these instances we have had available the corresponding malignancy arising in these areas. In none of these instances was the percentage succinate stimulation less in the tumor tissue than it was in the homologous control. In fact, the reverse appears to be the case. This is in direct contrast to the results reported when succinate stimulation is evaluated on the basis of increased oxygen uptake as found with the conventional manometric technics.

It will be noted that the absolute values for the endogenous TTC reduction are often less in the tumor as compared to the corresponding normal tissue. It should be remembered that particularly in human material, sections of "tumor tissue" actually contain but a small fraction of tumor cells infiltrating the stroma, which may be extensive due to desmoplastic reaction.

The endogenous reduction of TTC by liver, kidney and spleen slices from mice was found to be the same in the tissues from the control animals as compared to those bearing spontaneous mammary carcinomas. Similar agreement of values was also found when the TTC reduction in the presence of succinate was compared for these tissues in the tumor and non-tumor bearing animals. Parallel studies performed on C57 black mice with and without implanted sarcoma 180 failed to demon-

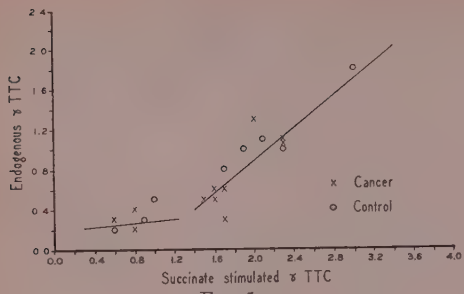

FIG. 1.

Plot of values of μg of TTC reduced per mg tissue in presence and absence of succinate. × = cancer tissue; ○ = control tissue.

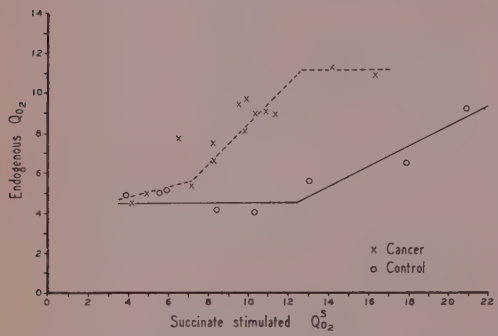

FIG. 2.

Plot of endogenous and succinate stimulated oxygen uptake (Q_{O_2}) for human and cancer tissues. Data of Roskelley *et al.*(1) and Rosenthal and Drabkin(2). ○ = control tissue; × = cancer tissue.

strate any change in the endogenous or succinate stimulated metabolism in liver and kidney slices. These findings are in accord with observations of Riehl and Lenta(9,10) who were unable to demonstrate a significant difference in the tissues of normal and tumor bearing mice in respect to total, malic and lactic dehydrogenase activity as measured by the Thunberg technique.

Discussion. In Fig. 1, we have plotted the values for the TTC reduction with and without succinate for a variety of control and tumor tissues. It appears from the distribution of the various values that a definite relationship obtains between the endogenous activity and the succinate stimulated activity. It is evident that the value for the endogenous reduction of TTC by a tissue is of greater significance in determining its response to succinate than is the benignancy or malignancy of the tissue. This observation suggests that both normal and tumor tissues tend to show a rather fixed relationship between dehydrogenase activity in the "resting" and stimulated state.

In an effort to evaluate this type of relationship in regard to endogenous and succinate stimulated Q_{O_2} values we have plotted these values as reported by Roskelley *et al.* (Fig. 2). It is evident that such a plot of the control tissues yields a curve very similar in shape to that obtained by the TTC technique, and is also indicative of a relationship between the endogenous Q_{O_2} value and the succinate stimulation. While the curve for the various tumor tissues is distinctly separated from the control except at the lower end of the curve it may be inferred that the succinate stimulated oxygen consumption of tumors is also a function of the endogenous respiration. The separation of the two curves as the Q_{O_2} values rise would indicate that a different member of the respiratory chain of enzymes functions as the limiting factor in tumors as compared to normal tissues. The similar type curve obtained for the oxygen consumption of normal tissues and the TTC reduction (dehydrogenase activity) of control and tumor tissues would indicate that it is not the dehydrogenase activity *per se* which is the limiting factor in the decreased oxidative stimulation of some tumors by succinate. In tumor tissues possessing a relatively high Q_{O_2} the limiting factor in succinate stimulation would be expected to occur in the cytochrome-cytochrome oxidase system. This is in complete agreement with Greenstein's demonstration that tumors show not only a lowered cytochrome c content but also the greatest disparity between cytochrome c and cytochrome-oxidase. From his data as well as our own it must follow that a disparity also exists between the cytochrome c and the components of the respiratory chain of enzymes preceding it; *viz.* dehydrogenases and flavoproteins.

Finally, it is particularly significant to note

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10. Riehl, M. A., and Lenta, M. P., *Cancer Research,* 1949, v9, 47.

that in regard to succinate stimulation of TTC reduction and oxygen uptake, tumor tissues behave not in an autonomous, unpredictable fashion, but rather in accord with equally rigid control as obtains in representative normal tissues.

Summary. Studies were made of the endogenous and succinate stimulated *in vitro* reduction of TTC by tissue slices from various human normal and cancer tissues as well as liver, kidney and spleen slices of control and tumor bearing mice. The per cent stimulation by succinate was not found to differ significantly in normal and tumor tissues. These findings were compared with previous studies of oxidative stimulation by succinate. It was also found shown that the presence of a tumor did not change the endogenous or succinate stimulated dehydrogenase activity of the liver, kidney or spleen of mice as measured by the TTC technique.

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Interrelationships of Vitamin B_6, Niacin, and Tryptophan in Pyridine Nucleotide Formation.* (18517)

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Since an increased excretion of niacin and some of its derivatives have been shown to result from the administration of tryptophan (1-4), tryptophan probably functions as a metabolic precursor of niacin. These studies have been implemented considerably by the work of Heidelberger *et al.*(5,6). It has also been observed that rats deficient in vitamin B_6 (pyridoxine) do not metabolize tryptophan normally as shown by the excretion of xanthurenic acid in the urine(7-10). Other workers(11,12) have observed that deficiency of combinations of various B vitamins besides pyridoxine also markedly interfere with tryptophan metabolism. Spector(13) reported that pyridoxine apparently had little effect upon the conversion of tryptophan to niacin or N-methylnicotinamide as measured by excretion of the expected metabolites. Recently it has been reported from this laboratory(14, 15) that dietary tryptophan appears to be more important in the maintenance of tissue pyridine nucleotides (DPN and TPN) than dietary niacin although one might expect the reverse to be true because of the presence of the niacin residue in DPN and TPN.

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In the present study the effect of a pyridoxine deficiency upon maintenance of liver pyridine nucleotides by dietary tryptophan has been investigated in an attempt to elucidate some of the existing problems concerning the function of pyridoxine in tryptophan metabolism. In addition the role of niacin in pyridine nucleotide maintenance has been further investigated.

Experimental. Two groups of male, weanling rats of the Sprague-Dawley strain weighing 40 to 50 g were used as experimental animals. One group of 30 rats (Group a) was given a pyridoxine-free and niacin-free ration (basal) and the other group of 12 rats (Group b) was given a niacin-free ration, *i.e.*, the basal ration plus adequate pyridoxine. The basal ration plus pyridoxine consisted of vitamin test casein 25, Salts IV(16) 4, corn oil 5, vitamin mix 2, and sucrose 64%. The 2 groups of rats were fed their respective rations and watered *ad libitum* for 9 months during which period 14 of the pyridoxine-deficient animals died with noticeable symptoms of the deficiency. No mortality was observed among the group receiving the niacin-free plus pyridoxine ration. At the end of the 9-month period the average weight of the remaining pyridoxine-deficient rats was approximately one-half that of the control group fed the niacin-free ration. After this period the rats in the pyridoxine-deficient group were separated at random into 3 groups and given the following supplements: Group 1a, none; Group 2a, 1.25 mg pyridoxine hydrochloride per 100 g ration (5 times the normal level); and Group 3a, 150 mg niacin per 100 g ration (100 times the level ordinarily fed to rats in synthetic rations). The group receiving the niacin-free plus pyridoxine ration for 9 months was divided into 2 groups and supplemented as follows: Group 1b, none; Group 2b, 1.35 g DL-tryptophan per 100 g ration (5 times the level fed in an 18% casein ration). The levels of the supplements fed were arbitrarily chosen in order to insure a large excess of each supplement. The supplemented rations were fed to the respective groups for 2 weeks after which the animals were sacrificed for determination of liver pyridine nucleotide concentrations by the method of Feigelson, Williams, and Elvehjem (17). Weight data were recorded throughout the feeding periods to observe if any correlation of weight with liver pyridine nucleotide concentration occurred.

TABLE I. Weight Changes During the Feeding Periods.

Group	Ration	Period (wk)	Avg change in wt per wk, g
a	— Pyridoxine — Niacin	36	+ 4.9
b	+ Pyridoxine — Niacin	36	+ 9.9
1a	— Pyridoxine — Niacin	2	0
2a	+ 1.25 mg % pyridoxine · HCl — Niacin	2	+24.2
3a	— Pyridoxine + 150 mg % niacin	2	— 5.7
1b	+ Pyridoxine — Niacin	2	+ 4.6
2b	+ Pyridoxine — Niacin + 1.35 g % DL-tryptophan	2	— 5.0

Results and discussion. The results of the weight changes during the experiment for the various groups are presented in Table I. The results are expressed as the average change in weight per week in order to compare the smaller supplemented groups with themselves rather than with the 2 large groups as a whole. It can be observed that the animals receiving no pyridoxine (Group a) gained an average of only one-half as much as the group receiving pyridoxine (Group b). Since the growth rate of both groups of rats had reached a plateau before the end of 36 weeks, the average weekly weight gain was lower than would be expected for the young growing rat. Nevertheless, the average weight of Group b was approximately double that of Group a at the end of this period.

After supplementation with the various substances given in the table for 2 weeks,

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17. Feigelson, P., Williams, Jr., J. N., and Elvehjem, C. A., *J. Biol. Chem.*, 1950, v185, 741.

TABLE II. Liver Pyridine Nucleotide Concentrations of the Groups After the 2-week Supplementation Period.

Group	Ration supplement	No. of rats	Liver pyridine nucleotide conc. (γ per g liver)
1a	0	5	807 ± 55*
2a	1.25 mg % pyridoxine • HCl	6	800 ± 54
3a	150 mg % niacin	5	1200 ± 150
1b	0	6	890 ± 60
2b	1.35 g % DL-tryptophan	6	1300 ± 73

* Stand. error of the mean.

definite changes in weight occurred in all the groups except the pyridoxine-deficient group receiving no extra supplement (Group 1a). The inclusion of pyridoxine in the ration of the pyridoxine-deficient rats (Group 2a) caused the expected sharp increase in growth, which was almost as great as the growth of young weanling rats fed a complete ration. When niacin was fed at a 160 mg % level to the pyridoxine-deficient rats (Group 3a) a significant decrease in weight occurred demonstrating a possibly toxic effect of niacin fed at this level. The rats receiving pyridoxine throughout the 9-month period and given no extra supplement for the extra 2-week period (Group 1b) continued to gain weight slowly. When 1.35 g % of DL-tryptophan was added to the control ration (Group 2b), the rats lost weight significantly during the 2-week supplementation period showing a toxic or imbalance effect of the high level of tryptophan.

In Table II are presented the results of the analyses for liver pyridine nucleotides of the various groups of animals after the 2-week supplementation period. The results are expressed as micrograms (γ) DPN plus TPN per gram of fresh liver. From the results of Groups 1a and 2a it appears that a pyridoxine deficiency has no demonstrable effect upon the production of liver pyridine nucleotides from dietary tryptophan. It is possible that enough pyridoxine was retained in the tissues of the rats from the weanling age to satisfy the enzymatic requirements for the conversion. However, some reduction in pyridine nucleotide concentration should have been observed if pyridoxine were highly important in the conversion of tryptophan. In an experiment not reported in this paper in which desoxypyridoxine was fed to both weanling and adult rats for several weeks, the liver pyridine nucleotide concentration was unaffected by the pyridoxine antagonist even when measured in the animals in a moribund state brought on by the antagonist. The animals, however, had not been depleted first of tissue pyridine nucleotides.

From the results of Group 3a it appears that niacin at the high level fed is able to spare liver pyridine nucleotides. Whether the niacin acts to spare the nucleotides by utilization in their synthesis or by interfering with the enzymatic breakdown of those already present is still open to question at this time. It is known that niacinamide is an inhibitor of the enzymes that destroy the pyridine nucleotides(18) as well as those that utilize them(19).

The results from Groups 1b and 2b indicate that tryptophan in excess of the amounts required for building protein can be converted to pyridine nucleotides rather than being completely removed by other metabolic pathways.

When the results in Tables I and II are compared there appears to be no positive correlation between weight changes brought about by the supplements in question and liver pyridine nucleotide concentration. In fact the 2 groups with the highest pyridine nucleotide concentrations (Groups 3a and 2b) actually lost weight during the supplementation period. The group gaining weight at the fastest rate (Group 2a) moreover has the same liver pyridine nucleotide concentration as the group neither gaining nor losing weight (Group 1a). Although the pyridine nucleotides are undoubtedly necessary for the normal functioning of an animal and therefore for growth, it is probable that the range of concentrations observed in these experiments

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19. Feigelson, P., Williams, Jr., J. N., and Elvehjem, C. A., *J. Biol. Chem.*, in press.

is actually in excess of the amounts necessary for adult rats. Pyridine nucleotide concentrations which could be correlated with growth changes would probably be obtained when the concentrations are lower than those required by the animal for normal function.

Summary. 1. It has been observed that a pyridoxine deficiency in the rat does not disturb the normal conversion of tryptophan to liver pyridine nucleotides to any demonstrable extent. 2. Niacin fed to pyridoxine-deficient rats spares liver pyridine nucleotide concentrations. 3. Tryptophan fed in excess along with normal dietary protein in a complete ration increases liver pyridine nucleotides concentrations above normal demonstrating that the excess metabolite can be utilized rather than simply degraded and excreted.

†One hundred g of vitamin mixture contained the following vitamins in a sucrose base: thiamine hydrochloride 10 mg, riboflavin 15 mg, pyridoxine hydrochloride 12.5 mg, calcium pantothenate 100 mg, biotin 0.5 mg, folic acid 1 mg, choline chloride 5 g, and i-inositol 0.5 g.

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Toxicity of Rubber Stoppers for Tissue Cultures.*† (18518)

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During studies on the development of a synthetic medium for animal cells in tissue culture(1-3), it was observed that chance contact of the synthetic feeding mixtures with the rubber stoppers used to close the culture tubes often killed the cells within 48 hours. The following experiments were undertaken in an attempt to reduce the toxicity of the rubber stoppers by various methods of chemical treatment and to test the relative toxicity of various types of rubber.

Methods. Chick embryo mesenchyme tissues, derived from skeletal muscle, were cultivated on the inner surface of standard Pyrex test tubes and supplied with a fluid synthetic mixture (No. 199), which was withdrawn and replaced at regular intervals. Full details of the culture methods and of the synthetic mixture have already been reported(1). Unless otherwise specified, the rubber stoppers that were tested were of the standard black laboratory variety, size No. 1, obtained from commercial supply houses. After chemical treatment (Tables I and II), 9 stoppers

TABLE I. Effect of Solvent Extraction on the Toxicity of Black Rubber Stoppers Tested in Tissue Culture.

Exp. No.	Treatment*	Survival (days)†
A	Control. Culture fluid not exposed to rubber	33
B	Control. Culture fluid exposed to untreated black stoppers	2
1	Hot ether (6 hr)‡	6
2	Hot acetone (6 hr)‡	3
3	Hot alcohol (6 hr)‡	3.2
4	Hot ether (2 hr), hot acetone (2 hr), hot alcohol (2 hr)‡	9.2
5	Cold ether (1 wk)§	8.6
6	Cold acetone (1 wk)§	2.5
7	Cold alcohol (1 wk)§	7
8	Cold ether (2 days), cold acetone (2 days), cold alcohol (2 days)§	7.3

* In each treatment, 9 new black stoppers were extracted with 250 ml of solvent, under the conditions specified, and were then boiled in 3 changes of distilled water.

† Each figure represents the average survival time of 6 cultures in each of the fluids tested.

‡ Extracted in a Soxhlet apparatus.

§ Solvent changed daily.

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TABLE II. Effect of Treatment with Hot Alkali and Acid on Toxicity of Black Rubber Stoppers Tested in Tissue Culture.

Exp. No.	Treatment*	Survival (days) †
A	Control. Culture fluid not exposed to rubber	33
B	Control. Culture fluid exposed to untreated black stoppers	<3
1	0.5 N NaOH, 0.5 N HCl	3
2	1.0 N NaOH, 1.0 N HCl	3
3	2.0 N NaOH, 2.0 N HCl	3
4	5.0 N NaOH, 5.0 N HCl	4.6
5	1.0 N NaOH, 1.0 N HCl (alkali and acid made up in 95% ethyl alcohol)	2
6	2.0 N NaOH, 2.0 N HCl (repeated twice)	3.8
7	7 days cold alcohol, then 2.0 N NaOH, 2.0 N HCl	2
8	2.0 N NaOH, 2.0 N HCl, then 7 days in cold alcohol	9.7

* In each treatment, 9 new black stoppers were boiled successively for 6-hour periods in 250 ml of alkali, acid and distilled water.

† Each figure represents the average survival time of 6 cultures in each of the fluids tested.

from each batch that was processed were placed in 500-ml Erlenmeyer flasks and autoclaved. The stoppers in each flask were then covered with 50 ml of Mixture 199 and left standing for 1 week at room temperature. At the end of this period, the fluids were removed from the stoppers and used as feeding solutions for the test cultures. The length of time the cells survived served as a measure of the effectiveness of the various treatments. All solvents and chemicals were of reagent grade.

Results. Exhaustive extraction of the stoppers with hot or cold solvents reduced the toxicity only slightly (Table I). The best results were obtained with ether, or with combinations of ether, acetone and alcohol. It was difficult, however, to remove these solvents from the stoppers, which became hard and brittle. Accordingly, solvent treatment was abandoned and various combinations of alkali and acid were studied (Table II). Prolonged boiling in NaOH and HCl, at concentrations sufficient to disintegrate the stoppers, did not appreciably reduce their toxicity. These attempts were also abandoned, therefore, and a comparative study was made of various types of rubber (Table III). The most suitable rubber tested was a gray product for pharmaceutical use prepared from crude rubber of virgin stock. This rubber was also slightly toxic, but the degree of toxicity was attributed mainly to the severity of the assay procedure. In any event, stoppers of this type have now been used successfully on many hundreds of cultures.

Discussion and conclusions. In most laboratories, it is customary to treat new rubber stoppers with hot alkali and acid in order to remove surface impurities. But in the present experiments, based on more than 500 cultures, stoppers that were strongly toxic in the beginning remained toxic even after the most rigorous treatment. It should be emphasized that the toxicity of rubber stoppers is much more evident in cultures maintained in synthetic media than in media containing such naturally-occurring ingredients as blood plasma, blood serum and tissue extracts. For this reason, extreme care should be taken to avoid toxic stoppers in all experiments in which protein-free media are used. And since animal cells cultivated in synthetic media serve as extremely sensitive test objects, the information reported here can almost certainly be applied to other biological systems.

TABLE III. Relative Toxicity of Various Types of Rubber Stoppers Tested in Tissue Culture.

Exp. No.	Type of rubber*	Survival (days) †
A	Control. Culture fluid not exposed to rubber	33
B	Control. Culture fluid exposed to ordinary black stoppers	2
1	Ordinary stoppers, source A	2
2	Ordinary stoppers, source B	1
3	Neoprene stoppers, source A	2.8
4	Neoprene stoppers, source B	3.8
5	Neoprene stoppers, source C	6.2
6	Red stoppers (virgin rubber)	2.2
7	Pure gum-rubber stoppers‡	12
8	Silicone-impregnated stoppers‡	12
9	Gray stoppers (virgin rubber) ‡	22

* With the exception of Nos. 8 and 9, which were washed only in distilled water, all types tested were first boiled successively for 6 hours in 2 N NaOH, 2 N HCl, and distilled water.

† Each figure represents the average survival time of 6 cultures in each of the fluids tested.

‡ Obtained from The West Co., Phoenixville, Pa. Stoppers in Exp. No. 9 specified as stock compound No. S-124.

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Evaluation of Tetrazolium as a Histochemical Index of Adrenal Cortical Activity.* (18519)

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In recent years, a number of specialized histochemical technics have been developed (1-4) in an effort to analyze the functional activity of the adrenal cortex more specifically than conventional procedures permit. These new tools provided an abundance of information enabling us to relate the adrenal cortex to different physiological and pathological processes. They were, however, limited in their scope, chiefly because of the static nature of the indices employed. It has proved difficult to measure objectively the dynamic processes of cell secretory activity, solely on the basis of criteria such as the intensity and character of the lipid distribution, or total cholesterol and ascorbic acid content. What appears to be lacking is some additional parameter which would provide a direct measure of the metabolic activity of the adrenal cortex and thereby enable us to interpret histochemical data more adequately. Unfortunately, the use of microrespiratory studies has as yet provided little or no metabolic evidence of this character. Other studies in our laboratory(5) led us to an exploration of the potential usefulness of 2,3,5-triphenyl tetrazolium chloride (TTC) for this problem. This reagent was selected because of a number of interesting properties. TTC is a colorless, water soluble compound which is transformed into a red, insoluble formazan by an active reduction process. In viable tissue slices, this chemical reaction involves an enzymatic reduction by intracellular reductases, such as the dehydrogenases, for which TTC acts as a hydrogen acceptor. The intracellular deposits of the colored formazan are clearly identified in frozen sections made after the tissues have been incubated in a TTC solution.

These properties of TTC have certain potential advantages with respect to the problem of adrenal cortical secretory activity. The reaction with TTC is carried out *in vitro* with viable slices of tissue and hence reflects the metabolic activity of the tissue at the time of study. The recent work of Kun and Abood(6), Antopol, Glaubach and Goldman (7), Ruttenberg, Gofstein and Seligman(8), Black and Kleiner(9), has demonstrated the validity of the reduction of TTC by living systems as a reflection of the metabolic activity of different cells. It is not yet known whether the reduction of TTC is an index of the activity of a specific dehydrogenase system or is an overall measure of different reductases. There is a growing body of evidence from various tissues that the extent of the deposition of the reduced TTC is roughly proportional to overall metabolic activity (unpublished observations). A more detailed analysis of the metabolic activity of a tissue, relating TTC reduction to specific enzymatic

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2. Greep, R. O., and Deane, H. W., *Ann. N. Y. Acad. Sc.,* 1949, v50, 509.

3. Sayers, G., *Physiol. Rev.,* 1950, v30, 241.

4. Selye, H., and Stone, H., *On the Experimental Morphology of the Adrenal Cortex,* 1950, C. C. Thomas, Springfield, Ill.

5. Zweifach, B. W., Black, M. M., and Shorr, E., Proc. Soc. Exp. Biol. and Med., 1950, v14, 848.

6. Kun, E., and Abood, L. G., *Science,* 1949, v109, 144.

7. Antopol, W., Glaubach, S., and Goldman, L., *Trans. N. Y. Acad. Sci.,* 1950, Series II, v12, 156.

8. Ruttenberg, A. M., Gofstein, B. S., and Seligman, A. M., *Cancer Research,* 1950, v10, 113.

9. Black, M. M., and Kleiner, I. S., *Science,* 1949, v110, 660.

systems, is made possible by varying the incubation medium by the addition of different substrates or selective enzyme inhibitors. It is hoped that the TTC procedure, when utilized in this fashion, may prove to be a valuable adjunct to the histochemical procedures at present available for evaluating the functional activity of the adrenal cortex. The present report is concerned with our initial application of the TTC technic to this problem.

Methods. Tissues were obtained from rats after stunning the animals by a blow on the head, and from dogs at operation under pentobarbital anesthesia. The organs were rapidly transferred into ice-cold saline, after which slices 1.0 to 2.0 mm thick were cut with a razor by hand, as for the conventional Warburg microrespiration studies. Special precautions were taken to cut the slices at right angles to the surface in order to include all 3 zones in each section. The tissue slices were briefly rinsed in chilled saline and placed in a 25 cc Erlenmeyer flask containing 5 cc of the incubation medium. This consisted of 3 parts of a 1% solution of 2,3,5-triphenyl tetrazolium chloride (TTC) to one part of physiological saline, the mixture being brought to pH 7.2 with 0.1 M PO_4 buffer. In order to provide an excess of TTC, a maximum of 2 to 4 slices (approximately 200 mg) of tissue to 5 cc of the incubation medium was routinely used. The flasks were covered with parafilm to prevent evaporation and gently shaken in a Warburg bath at 37.5°C for one hour in air. At the end of this period, the solution was decanted and the stained tissue fixed in 10% neutralized formalin. Exposure to direct sunlight was avoided so as to minimize its effect on the formazan pattern. For microscopic study, frozen sections varying from 5 to 15 μ in thickness were cut and mounted in glycerin. Duplicate sections were routinely counterstained with polychrome methylene blue, to allow better histological and cytological identification. Preparations stained with TTC gradually lose their color after repeated exposure to light and should be kept in the dark. In the present study, the sections were examined immediately after preparation and photographs (black and white, and kodachrome) were routinely taken within 24 hours after the sections were prepared. In selected experiments, the reduction of TTC was carried out in the presence of an added substrate, such as succinate, in order to accentuate the deposition of the formazan (3 parts 1% TTC—PO_4 buffer mixture to 1 part 0.2 M succinate). The histochemical intracellular staining pattern under these conditions was otherwise identical with that obtained in the absence of added substrate. However, an accentuation of extracellular deposition of formazan was noted, particularly in the junctional area between the glomerulosa and fasciculata.

Experimental results. 1. *Normal adrenals.* Following *in vitro* incubation with TTC, tissue slices become diffusely stained. An essentially similar type of diffuse staining was observed in the adrenals of normal mice, rats, guinea pigs, rabbits and dogs. Quantitative determinations indicated that the intensity of the TTC reduction by the adrenal would place it among the more active tissues in this respect. In terms of gamma TTC reduced/mg acetone dried tissue, the mean values for slices of dog kidney and adrenal are 4.2 and 1.7 γ/mg, respectively. The reduced TTC does not appear as discrete granules or crystals in the cytoplasm of the cell as it does in the kidney. Instead, the formazan is exclusively located in the lipid droplets of the cytoplasm, presumably because of its lipid solubility.

In the rat, the deposition was of uniform intensity throughout the 3 zones (Fig. 1A). In the dog, guinea pig and man, an especially prominent deposit of reduced TTC usually occurred in the zona glomerulosa. With conventional technics, there is no histologic distinction between the glomerulosa cells and the cells of the fasciculata where they lie directly adjacent to one another. However, with TTC, cells in the glomerulosa were readily identified since their lipid droplets were larger and were stained a more intense red than were those of the adjacent fasciculata (Fig. 1B, 1C), a further indication of a functional difference between these two zones. In general, it appeared that the cells of the reticularis were stained a more intense red than the inner fasciculata cells. In addition,

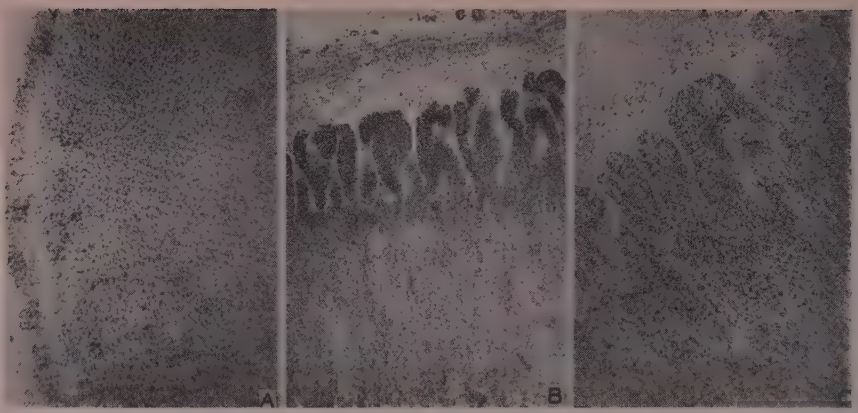

FIG. 1A. Adrenal cortex of normal rat after incubation with TTC for 1 hr. Note pale zone intermediate between glomerulosa and fasciculata in which no TTC deposition occurred. (Magnification 24×).

FIG. 1B. Adrenal cortex of normal dog stained with reduced TTC. The glomerulosa is prominent. (24×).

FIG. 1C. Adrenal cortex of normal dog under higher magnification (40×), showing distribution of reduced TTC within lipid granules of cells.

the lipid in these cells was usually more coarsely dispersed. Isolated cells, with staining characteristics similar to those of the reticularis cells, were often seen in the adrenal medulla, although direct anatomical connection with the cortex proper could not be seen. The cells of the adrenal medulla proper showed no significant intracellular deposition of formazan. There was, however, a variable amount of extracellular deposition of elongated and granular formazan crystals, as well as some purplish plaques throughout the medulla.

As can be seen in the accompanying photograph (Fig. 1A), the reduction of TTC in adrenals used for illustration was not always completely uniform, some areas being more heavily stained than others. The possibility of some of these variations being artifacts cannot be excluded at present. The staining of the fasciculata usually occurred fairly evenly, although in many sections there appeared to be a central zone in which the reduced TTC was sparse. This finding, however, was not a constant feature. These illustrations were selected to call attention to the occasional variations which were encountered.

Not infrequently, in tissues incubated with no added substrate, there was seen between the glomerulosa and the fasciculata of the rat adrenal a thin layer, several cells in thickness, which did not stain with formazan (Fig. 1A). While the cells of the glomerulosa characteristically have heavy lipid granules within the cytoplasm, the cells of the immediately adjacent zone contained little or no lipid (Fig. 1A). This intermediate TTC negative zone became more prominent under different experimental conditions, and, as will be shown later, was especially evident in the adrenals of hypophysectomized rats. In many sections of adrenal tissue there occurred a considerable deposit of reduced TTC extracellularly in the region of the sinusoids, the formazan appearing as coarse purplish plaques. This type of TTC reduction is most prominent in the glomerulosa and in the outer fasciculata. The endothelial cells lining the sinusoids of normal adrenals showed no intracellular TTC staining.

II. *Depleted adrenals.* A study was made of the tetrazolium reaction of the adrenal gland following two types of stress and during the acute response to the injection of ACTH. Six rats were each given 20 γ of ACTH† (with activity 8 times that of the Armour Standard)

† The ACTH used in these experiments was made available through the kindness of Dr. John R. Mote of Armour and Co., Chicago.

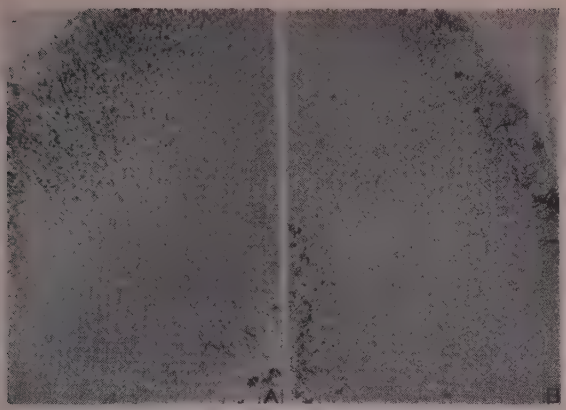

Fig. 2A. Adrenal cortex of rat stained with reduced TTC following the injection of 20 γ ACTH twice daily for 1½ days. The reticularis and inner fasciculata show pronounced depletion of lipid and are no longer stained with TTC. The formazan staining of the glomerulosa is unaffected. The unstained zone between the glomerulosa and fasciculata is not visible. (24×).

Fig. 2B. Adrenal cortex of rat stained with formazan following hemorrhagic shock for 2 hr. All three zones show a diminished capacity to reduce TTC, especially the fasciculata and reticularis. Some reductase activity is still present in the glomerulosa. (24×).

intramuscularly, twice daily. Three of the rats were sacrificed after 36 hours on this regime. Using TTC staining as a criterion, the most pronounced effect of the adrenocorticotrophic hormone was seen in the fasciculata. The cells of this zone showed an almost complete lack of TTC reduction, concomitant with a depletion of intracellular lipid. The cells of the reticularis continued to reduce TTC, with little or no reduction in their lipid content. The zona glomerulosa did not appear to be affected by the administration of this amount of ACTH (Fig. 2A).

Three rats were exposed to cold (1°C) for 72 hours and the adrenals immediately removed for *in vitro* study. In these animals, although the glomerulosa continued to stain, the two inner zones no longer reacted to TTC. The formazan staining indicated a marked falling off of reductase activity in both the fasciculata and reticularis. It is significant that the loss in TTC reduction was also accompanied by a depletion of the cytoplasmic lipid in the cells of the fasciculata and reticularis.

Several rats were subjected to hemorrhagic shock. The blood pressure was maintained at 40 mm Hg for 2 hours and the animals were then sacrificed. The adrenals in these rats showed the most profound reduction in formazan staining of the 3 stress situations employed here (Fig. 2B). The fasciculata and reticularis no longer reacted with TTC, and even the glomerulosa showed a significant reduction of its TTC activity. Here again, loss of the capacity to reduce TTC was paralleled by a loss of the preformed lipid from the cell.

III. *Adrenal atrophy.* (A) *Hypophysectomy.* Overall atrophy of the adrenal gland was brought about by hypophysectomy. A series of 12 rats, 30 to 40 days old, was studied at different stages following hypophysectomy.‡ These rats were allowed free access to both the stock rat pellets and the synthetic diet recommended by Shaw and Greep(10). They appeared well nourished

‡ We are indebted to the Hormone Assay Laboratories, Inc., Chicago, for the hypophysectomized animals used in the present study.

10. Shaw, J. H., and Greep, R. O., *Endocrinology,* 1949, v44, 520.

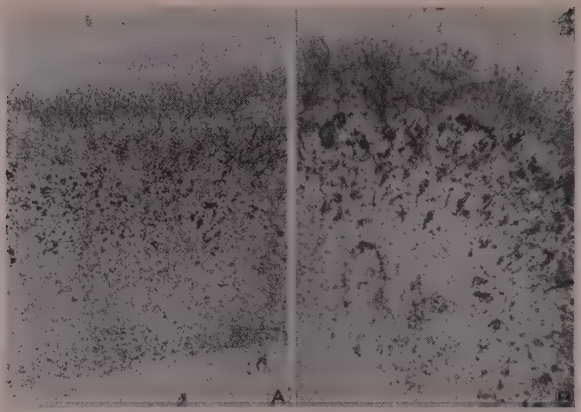

Fig. 3A. Adrenal cortex of rat stained with reduced TTC 11 days after hypophysectomy. The fasciculata and reticularis begin to show a lack of staining in many cells, despite the presence of lipid. The glomerulosa is unaffected. (24×).

Fig. 3B. Adrenal cortex of rat stained with reduced TTC 50 days after hypophysectomy. There is marked atrophy of the 2 inner zones, together with completely irregular and deficient TTC staining. Scattered reticularis cells continue to reduce TTC. The glomerulosa continues to reduce TTC actively. The irregular dark areas represent extracellular deposition of reduced TTC. (24×).

and gained weight throughout the study. The first set of adrenals was removed 8 days after hypophysectomy. At this stage, the glands as a whole had already undergone partial atrophy. There appeared between the glomerulosa and the fasciculata an unusually prominent zone in which there was no formazan staining and in which the cells contained only sparse lipid. The glomerulosa appeared normal in its TTC reaction, both in terms of the extent of staining and in the intensity of the formazan deposition. The fasciculata in these early hypophysectomized animals showed diminished, spotty staining. The reticularis appeared normal in regard to lipid content and continued to react with TTC as evidenced by its uniform staining.

A second set of animals was sacrificed 11 days postoperatively. In these adrenals the glomerulosa continued to stain well and actually appeared somewhat thicker than normal. The two inner cortical zones showed extremely irregular staining with formazan, despite the presence of lipid in many of these cells (Fig. 3A). Many of the fasciculata cells showed no staining with formazan. Others reduced TTC, but the intensity of staining varied widely in different areas, in contrast with the uniform staining of the normal fasciculata. Similarly, there was a marked variation in the lipid content of the cells of the fasciculata. A more uniform deposition of TTC was noted in the cortical cells immediately adjacent to the medulla. At 23 days after hypophysectomy, marked generalized atrophy of the two inner zones, the fasciculata and reticularis, was evident (Fig. 3B). The glomerulosa was no longer as prominent as it was at 11 days, but still continued to stain well with formazan. The intermediate zone between the glomerulosa and the fasciculata, which was readily discernible in the above 2 sets of hypophysectomized rats, was no longer visible. Actually, a clearly defined zona fasciculata could not be demonstrated by TTC. The innermost reticularis cells adjacent to the medulla continued to deposit appreciable amounts of TTC. In addition, considerable extracellular deposition of formazan now occurred, especially in the region immediately below the glomerulosa. At 50 days postoperatively, no uniform de-

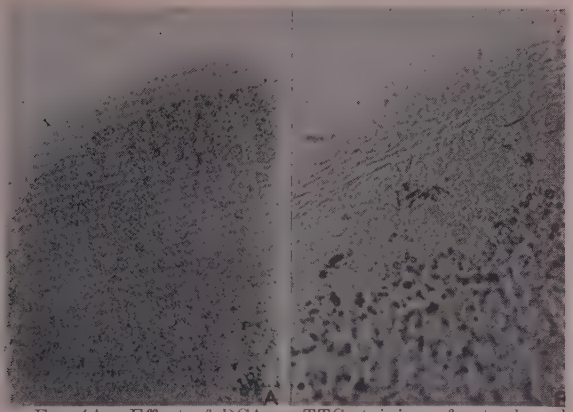

FIG. 4A. Effect of DCA on TTC staining of rat adrenal cortex. Complete suppression of reductase activity in the glomerulosa is evident, with no effect on the fasciculata and reticularis. (24×).

FIG. 4B. High power view showing TTC suppression limited to outer glomerulosa. (40×).

position of formazan occurred except in the zona glomerulosa. The reticularis continued to show scattered cells containing preformed lipid which was stained red with reduced TTC (Fig. 3B).

In sections which were counterstained with methylene blue there could be seen, interspersed throughout the two inner zones, irregularly shaped cells which did not stain with formazan but in which the cytoplasm was intensely basophilic. These cells were first noted as early as 8 days after hypophysectomy and could still be seen in adrenals removed 50 days after operation.

(B) *DCA inhibition of glomerulosa.* The usefulness of the TTC reaction as an index of functional activity of the adrenal cortical cells is best illustrated in experiments in which a particular zone of the adrenal is functionally depressed by the administration of a specific adrenal cortical hormone. Thus, a substance with mineralo-corticoid activity, such as desoxycorticosterone acetate, should, according to current concepts, selectively suppress the zona glomerulosa(11-13). Two 25 mg pellets of DCA were implanted subcutaneously in each of 12 rats. The rats were maintained on regular pellet rations and drinking water, and sacrificed after 14 to 30 days. As can be seen in the accompanying photograph (Fig. 4A), there was a specific effect on the zona glomerulosa, although no cytonecrosis was noted in the counterstained specimens. The cells showed a complete suppression of formazan staining, as well as a decrease of preformed lipid. The unstained cells of the glomerulosa were in sharp contrast to the contiguous cells of the fasciculata which continued to stain with formazan as under normal conditions (Fig. 4B). It is noteworthy that although some of the glomerulosa cells still contained preformed lipid, they did not reduce TTC. Work with specific enzyme inhibitors has shown that the enzymatic reduction of TTC may be inhibited without affecting the lipid content of the cell (unpublished observations).

IV. *Hypertrophy of the adrenal.* Hypertrophy was achieved by two procedures; first, by continued administration of 40 γ ACTH§ for 8 to 10 days, and second, by repeated sublethal trauma in the Noble-Collip drum. In both instances the adrenal gland underwent pronounced hypertrophy which was most evi-

11. Deane, H. W., Shaw, J. H., and Greep, R. O., *Endocrinology,* 1948, v43, 133.

12. Greep, R. O., and Deane, H. W., *Endocrinology,* 1947, v40, 417.

13. Selye, H., Stone, H., Timiras, P. S., and Schaffenburg, C., *Am. Heart J.,* 1949, v37, 1009.

§ Same preparation used previously.

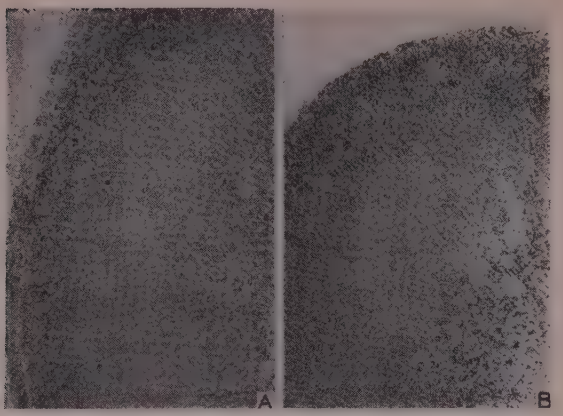

Fig. 5A. Hypertrophy of adrenal cortex of rat following chronic administration of ACTH. All 3 zones stain well with reduced TTC. Intermediate zone between glomerulosa and fasciculata still present. (24×).

Fig. 5B. Adrenal hypertrophy in rat subjected to repeated drum trauma. There is a considerable and uniform deposition of formazan throughout the gland. The intermediate unstained zone between glomerulosa and fasciculata is no longer demonstrable. (24×).

dent in the zona fasciculata (Fig. 5A, 5B). The cells in all 3 of the layers of the cortex contained considerable lipid. Following incubation with TTC, all of the cellular components uniformly reduced the TTC and were well stained. In adrenal hypertrophy, following ACTH administration, the unstained zone intermediate between the glomerulosa and the fasciculata was still visible. In adrenal hypertrophy following repeated sublethal trauma, the glomerulosa and fasciculata appeared fused so that distinction between the two zones was not possible on the basis of cytological features.

Discussion. It has been well established by previous workers(7-9) that the reduction of the colorless TTC compound to the insoluble red formazan, in tissue slice incubation studies of the type employed here, requires the enzymatic activity of living cells. It has also been found that the reaction is directly dependent upon temperature. Incubations carried out in air show a progressive reduction of TTC with time until a maximum is achieved after 60-90 minutes. The reduction is not carried out by injured or dead cells. Specific inhibitors which depress the metabolic activity of the cell also depress or completely abolish the ability of the cell to reduce TTC.

The application of this tool to studies of living cells was first made in experiments with seedlings(14) and then applied to a study of specific enzyme systems in unicellular organisms, such as yeast. Later, studies were carried out, incubating blocks of living tissue with TTC(15-17). It was not, however, until the work of Black and Kleiner(9) that use was made of the tissue slice technic as it is employed in microrespiration studies. These latter investigators(18) also found that the deposition of reduced TTC within the tissues could serve as a visible histochemical index to measure the activity of specific cells or cell components of different tissues. In a recent publication(5) from our laboratory, it was shown that TTC can be used as a histochemical index to detect metabolic alterations

14. Kuhn, R., *Ber. Deutsch. Chem. Ges.,* 1941, v74B, 949.

15. Mattson, M. A., Jensen, C. D., and Dutcher, R. H., *Science,* 1947, v106, 294.

16. Antopol, W., Glaubach, S., and Goldman, L., *Pub. Health Rep.,* 1948, v63, 1231.

17. Straus, F. G., Cheronis, N. D., and Straus, E., *Science,* 1948, v108, 113.

18. Black, M. M., Opler, S. R., and Speer, F. D., *Am. J. Path.,* 1950, v26, 1097.

in the renal proximal convoluted tubules of hypertensive patients and animals.

The capacity to reduce TTC under the aerobic conditions of these experiments (20% oxygen in air) is roughly proportional to the overall metabolic activity of the cell as determined by oxygen consumption measurements. Thus, when tissues are arranged in the order of their oxygen consumption, as measured by the conventional Warburg technic, their sequence is directly related to their capacity to reduce TTC. It should be emphasized that the TTC reaction is a measure of one or more hydrogen transfer mechanisms and therefore cannot be directly compared to oxygen consumption measurements which are a reflection of the total oxidative metabolism of the cell. It should also be recognized that cells probably carry out metabolic processes which do not involve reductase or dehydrogenase activities of the type measured by TTC. Changes in functional activity in these latter categories need not be reflected by changes in TTC reductase activity. The extent to which the reduction of TTC by the cells of the adrenal cortex is a reflection or a measure of the secretory activity of these cells is of primary interest. The secretory type of cell in the adrenal cortex contains numerous lipid granules of varying size. Such cells are seen in the normally functioning gland, as well as in the gland which has undergone hypertrophy. Cells which are hypofunctional, or undergoing atrophy, usually lose their lipid. We have found a definite parallelism between the deposition of formazan and the presence of lipid in the secretory type of cell. In normal and in hypertrophic adrenals, all of the zones of the cortex reduced TTC in a characteristic and uniform fashion. There were only two instances in which formazan staining did not occur, despite the presence of lipid in the cells. The first was seen in experiments with chemical inhibitors, where it was possible to obtain a specific suppression of TTC activity in the presence of intracellular lipid. Also, during the early stages of zonal inhibition following the injection of DCA, many of the cells in the glomerulosa were unable to reduce TTC, despite the presence of lipid granules. Since this hormonal effect apparently represents a condition in which the glomerulosa cells are undergoing atrophy and are functionally depressed, the absence of formazan staining is in accord with the concept of active TTC reduction as a reflection of the secretory activity of the cell.

Following hypophysectomy, a diminution or complete suppression of adrenal cortical secretory activity occurs in the fasciculata and reticularis of the rat(19,20). In our experiments, the development of a hypofunctional condition in these two zones was paralleled by a progressive failure of these cells to reduce TTC. The parallelism between adrenal cortical secretory function and the capacity to reduce TTC is thus again apparent.

Following acute stress or the discharge of the adrenal cortex by the administration of ACTH, a more complex situation exists. The cells of the fasciculata and reticularis discharge their lipid granules and, with conventional staining technics, appear depleted. This depleted state continues for a variable period of time before there is again clear-cut evidence of secretory activity as reflected by the appearance of intracellular lipid. The question remains as to what this morphological state represents in terms of functional activity. Is it a state in which the continued stimulation of the adrenal cortex results in a rapid and continuous discharge of the intracellular lipid secretions, so that no morphological evidence of such secretory activity is apparent, or does it represent a condition in which the cell is temporarily inactive and is slowly restoring its enzyme systems before it can again begin active secretion? The fact that these depleted cells do not actively reduce TTC would seem to favor the latter interpretation. This, however, does not exclude the possibility that the particular type of metabolic synthesis, in which the cells of the adrenal cortex are engaged at this stage, may not be reflected by the TTC reductase activity.

19. Deane, H. W., and Greep, R. O., *Am. J. Anat.*, 1946, v79, 117.

20. Selye, H., *J. Clin. Endocrinol.*, 1946, v6, 117.

The relative independence of the functional activity of the glomerulosa from that of the two inner zones of the cortex has been repeatedly demonstrated in the present TTC studies. Our data on adrenals of hypophysectomized rats indicate a continued staining of the glomerulosa, while the fasciculata and reticularis are losing this capacity. Likewise, the discharge of the fasciculata and reticularis by stress situations, such as cold, and by the administration of ACTH, is accompanied by no change in the capacity of the glomerulosa to reduce TTC. Finally, in experiments with DCA, the reduction of TTC by the glomerulosa was selectively suppressed, while the adjacent fasciculata and reticularis continued to stain. The demonstration of an intermediate zone between the glomerulosa and fasciculata was initially made by Sarason(21) and Simpson, Evans and Li(22), and also has been reported by Greep and Deane(2). We have been able to confirm the presence of such a zone in the rat adrenal with the TTC technic. This zone is clearly differentiated by its failure to reduce TTC. The precise significance of this finding is as yet unclear.

Mention has been made of the fact that the reduced TTC is regularly localized within the cytoplasmic lipid of the cells. This is in contrast to cells of other tissues which have no visible lipid granules in their cytoplasm and in which crystals of formazan are deposited as granules or needles. Furthermore, we have noted that active reduction of TTC is usually found in cells which contain lipid and which are in the process of active secretion. The question therefore arises as to the precise relationship of the TTC reductase activity and lipid formation. The lipid or lipo-protein complexes apparently do not possess the property of actively reducing TTC *per se*. It is our belief that the reduced formazan is localized in the lipid droplets because of its lipid solubility. Thus far, we have never observed, under normal conditions, adrenal cortical cells which are devoid of lipid and contain reduced TTC.

The TTC technic has permitted two types of evaluation of adrenal cortical activity. First, an overall estimation of the total amount of reduced TTC was made by photocolorimetric methods. These findings will be presented in a subsequent publication. Second, microscopic studies were made of the localization of formazan staining in specific zones of the adrenal cortex under various experimental conditions. At present, experiments are in progress with specific cortical hormones, such as Compounds B, E, F and S, in an attempt to determine their zones of origin. In addition, through the use of substrates and inhibitors, we are attempting to obtain more specific information concerning the precise enzyme systems which are related to the dehydrogenase activity of the cell as demonstrated by TTC reduction.

Summary. The application of the tetrazolium technic to a histochemical differentiation of the zonal activity of the various zones of the adrenal cortex is indicated. The functional activity of the cortical cells would appear to be paralleled by comparable changes in the ability of the cell to reduce tetrazolium. The implication of this type of approach to the problem of adrenal cytophysiology is discussed.

We are indebted to Mr. Jack Godrich for the microphotography in this study.

21. Sarason, E. L., *Arch. Path.*, 1943, v35, 373.

22. Simpson, M. E., Evans, H. M., and Li, C. H., *Endocrinology*, 1943, v33, 261.

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Occurrence of Lysozyme in Bird Egg Albumins. (18520)

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It has been reported by Fleming(1), Florey(2), Prickett, Miller and McDonald (3,4), Bradford and Roberts(5) and others, that lysozyme is widely distributed in animal tissues and fluids. Although none of these authors attempted to assign structural function to this enzyme, it is interesting to note the frequent occurrence of lysozyme. Quantitative data relative to the enzyme is not available, although a number of qualitative tests, some of which approximated quantitative procedures, have been made. Since Smolelis and Hartsell(6) described an accurate method for the determination of lysozyme from natural materials, it has been possible to accumulate more complete data.

Fleming(7) reported that lysozyme was present in the white of eggs from the thrush, the wagtail and the moorhen. Is it probable that the concentration of lysozyme in tissues or fluids is determined, in part, by the heredity of the species? A study of the lysozyme content of a number of egg albumins was carried out as a first step toward an answer to this question. Samples were obtained from chicken, bantam hen, guinea hen, goose, duck, turkey, quail, pheasant, chuckar partridge, horned lark, herring gull, and although not avian in origin, a number of turtle eggs.

Methods. The fresh eggs were broken and the whites separated from the yolks. These whites were then pooled and blended in a Waring blendor for 5 seconds. After mixing, the material was distributed into small test tubes, quick frozen and stored at −9°C. By handling in this manner it was possible to carry out the assays at convenient times. Earlier work had shown that no loss in the titer of egg albumin occurred after storage for several weeks. Lysozyme determinations were made in accordance with the method described by Smolelis and Hartsell(6). The results are presented in Table I. No calculated values are shown for the lysozyme contained in the goose or turkey egg albumins. Although these materials were soluble in phosphate buffer at pH 6.2, it was not possible to arrange a series of dilutions which resulted in turbidity values applicable to the standard curve. It may be that the lysozyme in the albumin of these birds was not free, therefore could not act as it would in pure form.

TABLE I. Lysozyme Titers of Frozen Bird Egg Albumins.

Source	No. of tests	Lysozyme, mg per ml* (avg)	Range
Bantam hen	5	6.9	6.4 -7.5
Quail	5	6.7	6.6 -6.7
Chicken	6	6.5	6.3 -6.9
Chuckar partridge	5	5.3	4.0 -6.0
Pheasant	7	3.4	2.9 -4.1
Guinea hen	5	3.1	2.8 -3.4
Duck	4	1.8	1.7 -2.0
Horned lark	4	1.0	0.9 -1.1
Herring gull	8	0.89	0.81-1.0
Goose	6	—	
Turkey	6	—	

* Expressed as mg of crystalline lysozyme per ml of albumin.

From these results it can be seen that an appreciable difference in lysozyme concentration exists between different bird egg albumins. However, a more complete survey is necessary before generalizations regarding species characteristic can be made. In attempting to determine lysozyme concentrations of turtle eggs a solubility problem was presented. In phosphate buffer, pH 6.2, a precipitate was formed. As an alternative method of assay for these albumins, agar cups were used. Previous experience had shown

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that the agar cup method for determining lysis was neither very accurate nor very consistent for the quantitation of lysozyme, nevertheless, it was applied to the albumins which did not lend themselves to turbidimetric assays.

Petri dishes containing 15 ml of nutrient agar were seeded with 4 ml of agar containing *Micrococcus lysodeikticus* cells. Nine-millimeter cups were formed in the agar and filled with the turtle egg albumin. For comparison, lysozyme dilutions were also prepared in the same manner. After 14 hours of incubation, the zones of inhibition surrounding the cups were measured. These were indicative of the bacteriostatic nature of the turtle egg albumin. Diffusion of some component, presumably lysozyme, in the turtle egg albumin prevented the growth of *M. lysodeikticus*. To further determine the bacteriolytic effect of this material upon the test species, another series of plates was prepared. These were seeded with ultraviolet, light-killed *M. lysodeikticus* cells and cups made as before. The amount of cells added was sufficient to impart a definite turbidity to the agar. After incubation, the zones surrounding the cups were measured. The clearing of the cells adjacent to the cup indicated that the material in the turtle egg albumin was lysozyme because the lysis of the dead cells is currently considered as a specific enzymatic reaction between lysozyme and its aminopolysaccharide substrate. Using this method, it was possible to demonstrate that undiluted turtle egg albumin caused a 20 mm zone of inhibition and a 17 mm zone of lysis. A 1:10,000 dilution of crystalline lysozyme caused a 20 mm zone of inhibition and a 14 mm zone of lysis. In this manner lysozyme was demonstrated in turtle egg albumin.

These results indicate the distribution and quantitative variation of this enzyme in egg albumins. There is variation between species of birds in the lysozyme content of their eggs.

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Relation of Clearance and Distribution of Injected Glutathione to Protection Against Radiation Injury.* (18521)

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In a series of studies on the effect of glutathione (reduced) on radiation injury it has been shown that this sulfhydryl tripeptide will protect significantly when administered before irradiation(1,2), that the radiation dosage mortality curve is significantly shifted to the right above an LD_{20}(3), and that there is histologic evidence of more rapid regeneration of the hematopoietic organs in the glutathione treated mice(4). With the publication of evidence by Forrsberg(5) that bacteria, skin, and hair follicles could be protected by the local action of cysteine, the question naturally arose as to whether glutathione protection of the irradiated animal arose from selective concentration of reduced glutathione in radiosensitive tissues essential to survival. Accordingly studies on the clear-

* Opinions and assertions are those of the authors and are not to be construed as reflecting the policies or opinions of the Navy Department at large.

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ance rate and distribution of injected reduced glutathione were performed.

Materials and methods. All solutions of reduced glutathione and cysteine hydrochloride were prepared as a 10% solution in sterile distilled water, and the pH was adjusted to 6.5 with 10% sodium hydroxide. Blood clearance studies were performed on mongrel dogs. These dogs were injected intravenously through 18 gauge needles into a vein of the foreleg. The injection time was maintained constant at 15 seconds. Blood was withdrawn at stated intervals after injection and the levels of reduced glutathione in the blood were determined by the iodometric method of Woodward and Fry(6). White Swiss male mice, weighing 22-28 g, were used in studying the disappearance time of injected reduced glutathione from the whole body of the mouse. The glutathione was administered in single subcutaneous injections (5 mg/g mouse) with a 24 gauge needle into the backs of the mice. For this study the whole body was weighed and then ground with 100 ml of distilled water for 4 minutes in a standard Waring blendor. Powdered, solid CO_2 was used to maintain the temperature at approximately 2°C. Protein-free filtrates of the tissue homogenates were then made as prescribed by the method of Woodward and Fry(6). With this method, 90-95% of added reduced glutathione could be recovered from the tissue homogenates. For the tissue distribution studies, white Swiss male mice were injected subcutaneously and Sprague-Dawley rats were injected intravenously with 5 mg/g of animal. These determinations were made by cutting the organ into fragments which were placed in a micro Waring blendor containing dry ice and 50 ml of distilled water. The temperature was maintained at approximately 2°C with powdered dry ice while the tissues were being macerated. Protein-free filtrates were then made according to the method of Woodward and Fry(6). Total glutathione levels were not made because the published methods do not yield consistent results(7,8).

Results. The level of reduced glutathione in the blood of 4 dogs was determined daily under fasting conditions for one week prior to the determination of the clearance rate of intravenously injected reduced glutathione. The mean concentration of reduced glutathione in the whole blood of the 4 dogs was 35.7 ± 7.2 mg/100 ml of blood. The mean blood glutathione of each of the four dogs respectively, was 36.0, 36.1, 33.5, 34.7 mg/100 ml of blood. In Fig. 1 is plotted the rate at which intravenously injected reduced glutathione disappears from the blood of dogs. The first point on each curve is calculated by assuming a uniform distribution of the intravenously injected glutathione in a blood volume equal to 7% of the body weight.

Following the injections of glutathione and cysteine hydrochloride at pH 6.5 in the stated doses, systemic reactions occurred. Respiration became more rapid, the pulse accelerated, and the dogs became excited. The symptoms subsided within five minutes. From Fig. 1 it is apparent that the non-protein reduced glutathione and cysteine levels had returned to the normal range within 30 minutes after injection.

The disappearance of subcutaneously injected glutathione from the body of the whole mouse was studied as follows: 5 mg of reduced glutathione per gram of mouse was injected subcutaneously. This amount occasionally killed a normal mouse. Many of the mice became cyanotic and shivered for 10-15 minutes after injection. The total amount of reduced glutathione remaining in the body of the mouse was determined at various time intervals after injection. In Fig. 2 is seen the rate at which the reduced glutathione disappeared from the body of the mouse. Each point on the curve is based on the average content of glutathione in the bodies of three mice sacrificed at stated times. The first point in Fig. 2 is the calculated value based on the amount of glutathione injected plus the mean glutathione level of the body of normal mice. The whole body gluta-

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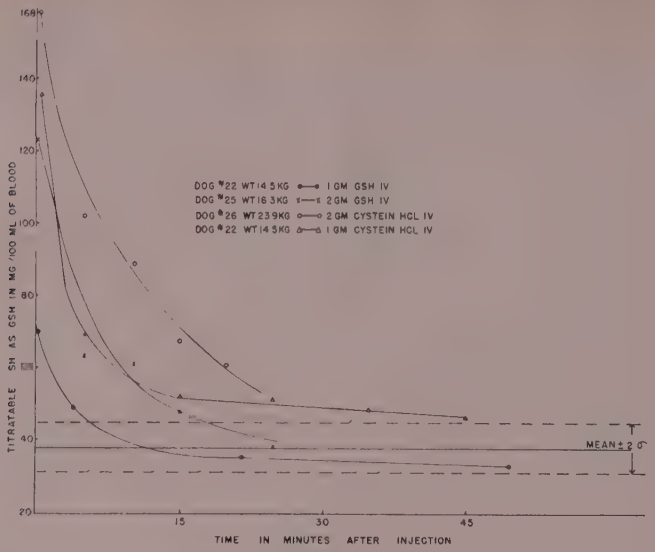

FIG. 1.

Blood levels of non-protein sulfhydryl as reduced glutathione (GSH) in mg/100 ml of whole blood following injection of both glutathione (pH 6.5) and cysteine (pH 6.5) intravenously in dogs.

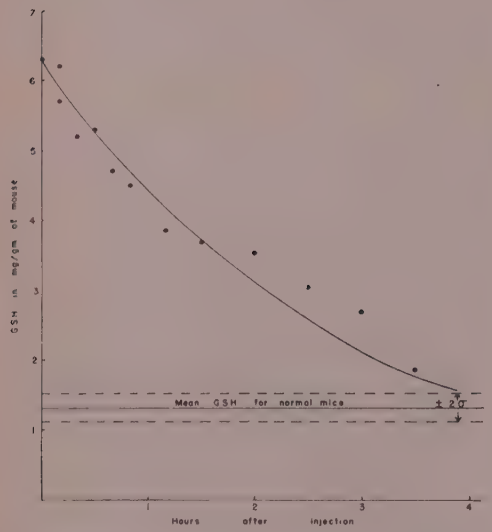

FIG. 2.

Disappearance rate of reduced glutathione (GSH) from the body of the mouse after subcutaneous injection 5 mg/g of mouse. Each point represents the average of three mice.

thione content remains two times above the normal levels for mice over two hours after a single subcutaneous injection of 5 mg per gram of mouse. It took approximately 3 hours for the reduced glutathione content of the whole body of the mouse to return to the normal range.

Following the establishment of the general period of time that the content of reduced glutathione in the bodies was elevated, the distribution of reduced glutathione in various tissues of mice and rats was determined. In mice, the concentration of reduced glutathione in the blood, liver, and spleen was determined 45 minutes after the injection of 5 mg of glutathione per gram of mouse. In rats, the distribution of glutathione in the blood, liver, spleen, testicles, small intestine, thymus, muscle, and kidney was determined 30 minutes after the intravenous injection of 5 mg/g of rat. The concentrations compared to normal concentrations in these species are tabulated in Table I. The mean value of the organs for 6 normal animals and 6 injected animals is compared in this table.

The concentration of reduced glutathione in the liver, spleen and kidneys was definitely increased. The concentration in the thymus and the small intestine was questionably increased. The measurement of glutathione in the bone marrow of the rat was attempted, but satisfactory preparations were not achieved and the great variability in the re-

TABLE I. Distribution of Injected Reduced Glutathione in the Tissue of Mice and Rats. Each value is the mean of 6 animals in mg/100 g of tissue.

Mice	Controls	45′ after 5 mg per g of mouse subcut.
Blood	39	44
Liver	344	435
Spleen	275	322
Rats		30′ after inj. 5 mg/g i.v.
Blood	41	48
Liver	338	476
Spleen	288	376
Testicles	293	292
Small intestine	278	295
Thymus	276	312
Muscle	56	63
Kidneys	205	310

sults prevented the accurate determination of differences in concentration of injected and control animals.

Discussion. The distribution and disappearance of reduced glutathione after intravenous and intraperitoneal injections has been previously reported by Braier(9). The results reported herein are in good agreement with his initial work. Both intravenously and subcutaneously injected glutathione tend to concentrate in specific tissues (liver, spleen, and kidney), rather than to be uniformly distributed throughout all tissues. In previous studies reporting the protective effect of glutathione(1-3) the period of irradiation coincided with the period of elevated glutathione concentration in the body at large, and these specific organs.

Adequate concentration of sulfhydryl compounds have been shown to protect unicellular organisms and locally irradiated skin and hair follicles(5). These findings, the ability of the animals whose spleen has been shielded to survive lethal doses of X-ray(10), and the high concentration of glutathione in the spleen, thymus and liver constitute an impressive sequence of events suggesting that glutathione may protect against whole body radiation by protection of some cellular or humoral element in the spleen, thymus, or liver which in turn can accelerate regeneration of the bone marrow which is essential for survival in the dose range where the sequelae of pancytopenia are the main causes of death. Unfortunately glutathione levels in the bone marrow are not available, but should these be high it would suggest that glutathione might protect the hemopoietic system by concentrating at the sites of hemopoiesis (bone marrow in the dog; spleen and bone marrow in the mouse).

Indirect influences cannot be excluded because glutathione has been shown to have other properties that might indirectly influence recovery from various stresses. For example, it has been shown that glutathione discharges the adrenal ascorbic acid(11) and will protect against potassium intoxication (12).

Summary and conclusions. 1. The clearance of intravenously injected reduced glutathione from the blood of dogs is rapid (< 30 min.). 2. The disappearance of subcutaneously injected glutathione from the body of the mouse is much slower (> 3 hours). 3. The distribution of injected reduced glutathione is not uniform throughout the body. Of the organs studied it concentrates to a considerable extent in the liver, spleen, and kidneys.

4. These experiments suggest that concentration of glutathione at sites vital for survival may be the prime factor in glutathione protection of the irradiated mammal. Whether the protection arises from preventing radiation death of essential cells or whether the protection is mediated through protection of humoral factors essential to orderly hemopoiesis remains unanswered.

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A Method for the Demonstration of Tissue Antibody.* (18522)

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A new technic is presented for the demonstration of tissue agglutinins before their appearance in circulating body fluids. Although the capacity of tissues to hold homologous antibody was shown by Kahn(1), no satisfactory method has heretofore been presented for the demonstration of tissue antibody. The present method was devised during a series of investigations concerning formation of antibodies *in situ*.

Preparation of tissues and organs. Small pieces of subcutaneous tissue from immunized mice were removed with forceps and ocular scissors, spread rapidly and thinly on clean glass slides and air-dried. The tissue was ringed with vaseline, homologous antigen added and the preparation sealed with a sterile coverslip. This direct spreading or smearing method for subcutaneous connective tissue was designed previously as a method for histological investigations. Organs to be examined were cut with a sharp scalpel or razor and the cut surface touched directly to a glass slide. The imprints thus formed were treated in the same manner as the subcutaneous spreads. All smears were made in duplicate in order to correlate immunological and histological studies. The slides for histological study were not sealed with vaseline but after air-drying were stained with May-Grünwald-Giemsa stains. Preparations from control groups of non-immune animals were run simultaneously with animals in various stages of immunization.

Preparation of antigens. Bacteria grown on a 0.5% dextrose nutrient agar base for 24 to 36 hours, were washed off with 0.5% HCHO in physiological saline and incubated overnight to insure sterility. When the bacterial suspensions were found to be sterile the organisms were washed in 0.025% HCHO dissolved in 0.85% NaCl and then centrifuged. This procedure was repeated 3 to 5 times. After washing, the organisms were diluted to a concentration of approximately 4 billion per milliliter. This was most conveniently obtained by allowing the original mixture to stand for 3 to 4 days at 4°C, after which time most clumps of organisms had settled out under gravitational influence and only the freely suspended and dispersed organisms remained. The suspension was examined microscopically to insure absence of bacterial aggregates.

Cellular antigens such as the sheep erythrocytes were washed and prepared in a 2% suspension in 0.85% NaCl and checked microscopically before use, for the presence of lysed stroma. The presence of lysed stroma in excessive numbers favors rouleaux formation and suspensions containing them are unreliable.

Agglutination technic. Two or 3 drops of antigen suspension plus equal volumes of physiological saline were added to the air-dried loose connective tissue and imprints. These preparations were incubated at 37°C for 1 to 2 hours and then examined microscopically. Final readings were made after remaining at 4°C overnight. When agglutinating antibody was present, well defined bacterial aggregates were seen adhering to the cells (Fig. 2, 4, 6). No such phenomenon was observed in similarly treated preparations taken from non-immunized control animals (Fig. 1, 3, 5). Positive results may be induced by microscopic fat globules. Such aggregates of bacteria differ from true agglutinations in that they are smaller, do not adhere tightly and uniformly and are found only in contact with the globular fat particles.

Discussion. The technic above can be used for the qualitative demonstration of agglutinating antibodies against *Salmonella ty-*

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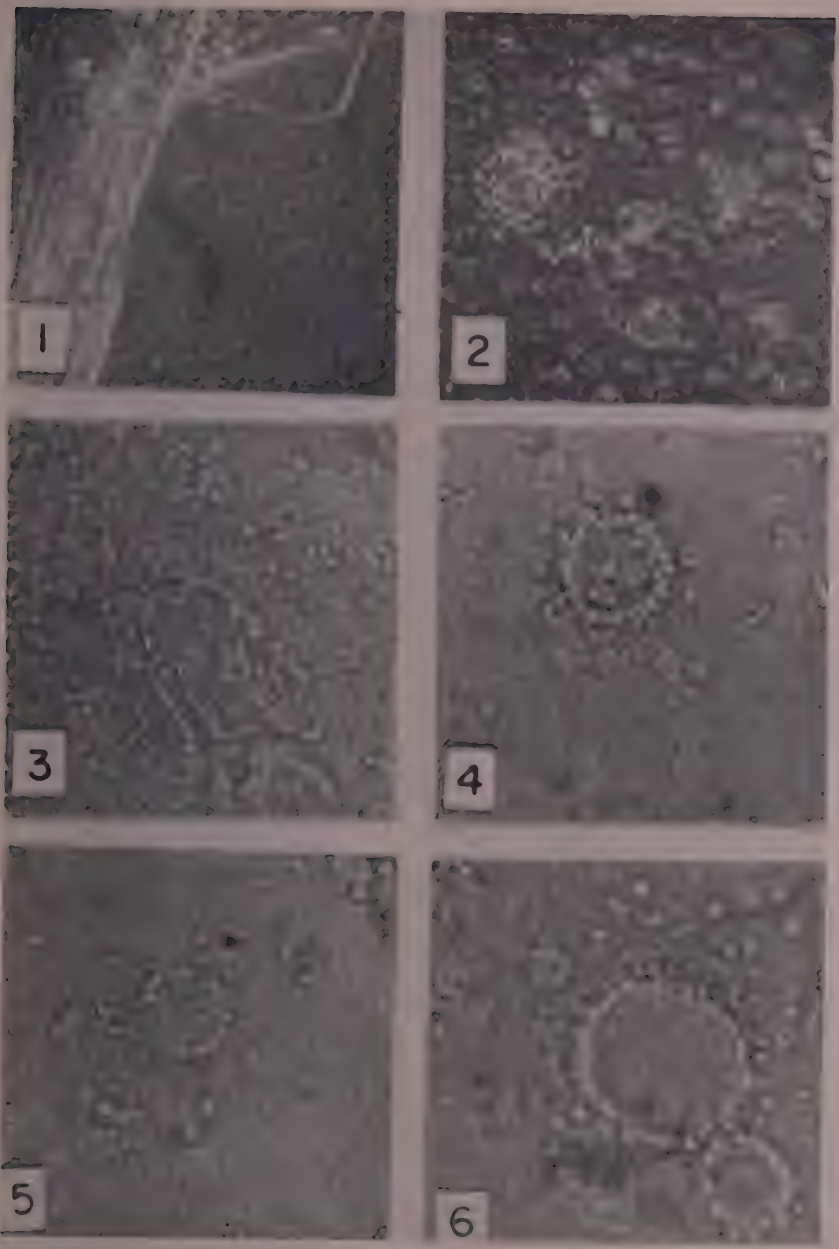

FIG. 1. Spread of loose subcutaneous tissue from non-immunized mouse. *Salmonella typhimurium* antigen added. Collagenous fibers and fibroblasts prominent with nuclei of latter visible among the fibers. Bacteria uniformly dispersed around tissue. No agglutination. Phase photomicrograph. Bright M contrast phase B, ×1000. Panatomic X film without filters.

FIG. 2. Spread of loose subcutaneous tissue from mouse 6 days after injection of approximately 9,000,000 *Sal. typhimurium organisms,* homologous antigen added. In the central part of the picture bacteria are agglutinated tightly to surface of lymphocytes while

intracellular areas show freely dispersed antigen particles. Phase photomicrograph conditions as given in Fig. 1.

FIG. 3. Spread of loose connective tissue from non-immunized mouse plus *Sal. typhimurium antigen*. Note uniform dispersion of antigen and prominent fibroblast cytoplasm and nuclei. No agglutination. Light microscope, ×2000.

FIG. 4 Spread loose subcutaneous tissue from mouse immunized 6 days previously with *Sal. typhimurium* antigen plus homologous antigen. Note "cartwheel" agglutinated bacteria around lymphocytes which is lacking in adjacent polymorphonuclear leucocytes. Light photomicrograph same as Fig. 3.

FIG. 5. Spread of loose subcutaneous tissue from non-immunized mouse plus *Sal. typhosa* antigen. Observe sharply defined cell outlines exhibiting none of the polar "cartwheel" attraction noted in Fig. 4. Light photomicrograph same as Fig. 3.

FIG. 6. Spread loose connective tissue of mouse immunized 6 days previously with *Salmonella typhosa* with homologous antigen added. Note strong polar attraction for bacteria by large lymphocyte in center of field. This effect is not shown by smaller more mature lymphocyte seen directly above. Light photomicrograph same as Fig. 3.

phosa, S. typhimurium, Pseudomonas aeruginosa and sheep erythrocytes. Agglutinating antibody to these antigens was detected in the loose connective tissue at the site of injection before it appeared in the blood. No antibody was found in the livers or spleens of immunized mice and rabbits before it began to circulate.

Although the technic described here is not adapted to quantitative measurement of antibody, it has value in detecting the presence of antibody in tissues and cells. It can, therefore, be used in investigations designed to secure information concerning various problems of immunophysiology.

The findings reported here support the previous observations that antibodies can be formed locally and also that the lymphocytes contain specific homologous antibodies(2,3). It is possible that this method for detecting cellular antibody could be adapted to practical problems concerned with the diagnosis of certain diseases, *e.g.* brucellosis in which circulating antibody may be extremely low.

Summary. A direct slide agglutination technic is described for determining the presence of agglutinating antibody in air-dried films of tissues and cells. This method has proved to be of value in correlating the appearance of antibody with cytological changes occurring at the site of antibody production.

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Maintenance of Denervated Taste Organs in Adult *Triturus v. viridescens*. (18523)

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Contrary to the widespread belief(1-6) that taste organs are dependent upon the trophic action of the gustatory nerve for development and maintenance, it has been demonstrated that amphibian taste organs can develop independently(7,8) and denervated organs can be maintained for at least 6 months (9). The present experiments not only

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support this, but extend the investigations to show that denervated taste organs can exist for at least 12½ months.

To effect permanent removal of gustatory nerve influence, autoplastic grafts of tongue tip to orbit (eye and eyelids removed) were made in adult *Triturus v. viridescens* under light chloretone anesthesia. Before recovery from the anesthetic, the animals were wrapped in wet absorbent cotton and kept in moist chambers in the icebox for 48 hours to prevent accidental removal of the graft. They were then placed in aquaria where the graft would be bathed by water at all times. In 90% of the cases circulation was re-established within one week and the grafts "took." In the process of establishment, varying amounts of resorption occurred. Also during the progress of the experiment the grafts tended to decrease in size. Animals were sacrificed according to the following schedule: daily for 30 days; on the 46th day; each month for 11 months; and at 12½ months after operation. Histological sections of 64 heads were examined for taste buds on the grafts.

During the first week after operation the tongue graft epithelium became thin and flattened, and there was considerable edema and necrosis in the epithelium and underlying tissues. The extent of these reactions varied in the different grafts. Two days after operation, 26 taste organs were present in one graft. Other clumps of cells which may be distorted organs were also noted. Between the 2nd and the 14th day, no taste organs were visible in the specimens examined.

During the second week after operation there was a progressive thickening of the epithelium. Many mitotic figures were evident. By the 14th day it compared favorably in thickness with normal tongue epithelium. In a graft of 14 days, 98 taste organs of normal appearance were counted. This was a larger graft than the 2 day specimen previously mentioned.

Mintz and Stone(9) stated that they could find no taste organs in the few specimens killed from 18 to 28 days after similar operation, and queried whether these were exceptions or indications of a significant period in the history of post-operative changes. In the experiments reported here a somewhat similar set of circumstances was found in specimens of 15-23 days. Though taste organs were present in grafts of 15, 16, 18-22 days, many of these were poorly defined organs. No taste organs were found in specimens of 17 or 23 days. To illustrate, in a 23 day graft, with no visible taste organs, the epithelium was thin in some areas, resembling a much earlier stage. The graft was edematous and gave a pale staining reaction, though the rest of the head appeared normal. In a 25 day graft, 104 organs were counted. From the 24th day on, taste organs were present in all cases.

The interpretation offered here is that after thickening of the epithelium to normal proportions, there is a period of reorganization involving the reappearance of the taste organs. It seems reasonable to suspect that taste organ cells are present all of the time but not in the normal arrangement of organs. When the data from Mintz and Stone's work and the present experiments are compared, it is seen that there is a great variation between different animals regarding the time of reappearance of discrete organs, but that toward the end of the first month many taste organs are present. Perhaps a correlation might be found between the variation in the amount of edema and necrosis after transplantation and the length of time necessary for taste organ reorganization. That the reappearance of taste organs can have nothing to do with gustatory nerve induction is evident, since it is inconceivable that such nerve fibers could be present. No attempt has yet been made to determine the presence or absence of other types of nerve fibers.

After this period, taste organs existed for many months (51 organs in one 12½ month case). Mitoses in taste organ cells have been observed as late as 8 months after operation. Again it is evident that taste organs can persist for many months without gustatory nerve influence.

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Incorporation of Glycine-2-C^{14} into Purines of Pentose Nucleic Acid and Desoxyribose Nucleic Acid.* (18524)

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The concept of the biochemical stability of desoxyribose nucleic acid (DNA) in non-growing tissues has gained wide acceptance. This concept has been supported by many studies involving chemical analysis, which have been admirably summarized by Davidson and Leslie(1). Moreover, Brown and co-workers(2) have reported that labeled adenine is not incorporated into DNA purines. Hevesy (3) has reviewed the work with P^{32}, which is incorporated to only a very slight extent into the DNA of resting tissues. In striking contrast to these results, we have reported (4,5) the extensive incorporation of glycine-2-C^{14} into the DNA purines, even in the case of adult rat liver, which is characterized by its low rate of mitosis. A considerable incorporation of small-molecule precursors into DNA purines has been reported independently by Elwyn and Sprinson(6) with glycine-2-C^{14} and with serine-3-C^{14} and by Totter *et al.*(7) with labeled formate. These workers, however, isolated their DNA from mixed tissues, some of which have a higher growth rate than liver. It has been demonstrated in birds by Sonne, Buchanan, and Delluva(8) that glycine is incorporated into positions 4, 5, and 7 of uric acid, and formate, in carbons 2 and 8. Karlsson and Barker(9) have shown specifically that carbon-2 of glycine becomes carbon-5 in uric acid. That these conclusions also applied to the rat was demonstrated by Heinrich and Wilson(10) with adenine and guanine isolated from mixed nucleic acids.

Since it is well known that glycine is converted into formate, also a purine precursor, it appeared to be of importance to determine whether the incorporation observed in our experiments(4,5) was that due to formate, which might be thought to exchange more readily with the two ureide carbons, or to glycine itself. Accordingly, the amount of radioactivity in carbon-5 of adenine and guanine derived from the PNA and DNA of liver and tumor was determined.

Experimental. Rats, bearing multiple transplants of Flexner-Jobling carcinoma, were given a single dose of glycine-2-C^{14} by stomach tube. After the appropriate time, the animals were sacrificed and the nucleic acids were isolated as the barium salts as described elsewhere(5). The DNA samples were contaminated with less than 10% of PNA. The nucleic acids were hydrolyzed and the purines isolated and purified by a modification of the ion-exchange chromatography method of Cohn(11). The specific radioactivity of the pure bases was determined, and they were hydrolyzed to glycine (12). The formaldehyde that was liberated

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Animal	Sample	Sp. act. purine	Sp. act.* CH_2O calc'd	Sp. act. CH_2O found	% in carbon-5
G 24, 25	Liver PNA, guanine	1.1	5.1	3.1	60
G 24, 25	" DNA, "	0.3	1.4	1.1	75
G 6, 7	" PNA, "	1.9	8.3	4.5	54
G 6, 7	" DNA, "	0.77	3.5	2.2	63
G 6, 7	Tumor PNA, "	6.1	27.0	16.0	59
G 6, 7	" DNA, "	4.8	22.0	15.0	68
G 26	Liver PNA, "	2.1	9.3	3.4	36
G 26	" DNA, "	1.2	5.4	2.6	48
G 26	" PNA, adenine	4.7	21.0	8.0	38
G 26	" DNA, "	10.1†	45.0	13.0	29

Specific activity of the administered glycine was 10,000 cpm per μg.

* This would be the specific activity of the formaldehyde if the entire radioactivity of the purine were in carbon-5.

† Since this DNA adenine had a higher specific activity than that of the corresponding PNA adenine, it was subjected to further chromatography. No significant change in the specific activity was observed.

G 24, 25 were 2 female 220 g rats, sacrificed 48 hr after 1.87 mg of glycine was given to each. The livers were pooled.

G 6, 7 were 2 female 166 g rats bearing 8-day implants of Flexner-Jobling carcinoma. They were sacrificed 18 hr after administration of 1.49 mg of glycine to each and the tissues were pooled.

G 26 was a 250 g male rat, sacrificed 18 hr after administration of 2.75 mg of glycine.

from the glycine by ninhydrin is derived from carbon-5 of the purine base. The specific activity of the formaldehyde was determined by colorimetric analysis of an aliquot with chromotropic acid, and by direct counting of the dimedon precipitate (carrier added). The specific activity of the formaldehyde was compared with that of the purine, and the results are shown in Table I.

Results and discussion. The data clearly show that a very appreciable per cent of the C^{14} in the purine bases was derived from glycine itself. Presumably of the remainder, most was incorporated as formate into the 2 and 8 carbons. The significant fact is that the results are essentially the same for the guanine and adenine derived from the DNA and PNA, and for both tumor and liver. Thus it is clear that even in the DNA of resting tissue the incorporation of glycine into the purines represents not a simple exchange of ureide carbons, but that exchange or synthesis of the main carbon skeleton of the purines has taken place.

Thus, it must be concluded that at least two pathways of DNA synthesis exist. One involves the incorporation of pre-formed adenine together with desoxyribose and phosphorus(2,3) into the nucleic acid. The other, and perhaps preponderant route, involves the incorporation of small-molecule precursors of purines into a skeleton to which the desoxyribose and phosphorus have already been affixed. This incorporation must take place in such a way that the total quantity of DNA in resting tissue remains essentially static(1). A possible intermediate in this process might be 4(5)-Amino-5(4)-imidazole carboxamide (13-15).

Summary. The extensive incorporation of glycine-2-C^{14} into the purines of PNA and DNA has been shown in both cases to take place largely by an actual incorporation of the glycine molecule, and not by a simple exchange of ureide carbons. The main implication of this finding is that at least two pathways of DNA synthesis must exist.

13. Shive, W., Ackermann, W. W., Gordon, M., Getzendaner, M. E., and Eakin, R. E., *J. Am. Chem. Soc.*, 1947, v69, 725.

14. Ravel, J. M., Eakin, R. E., and Shive, W., *J. Biol. Chem.*, 1948, v172, 67.

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Comparative Studies on Toxicity of a New Streptomycin and Streptomycin Sulfate.* (18525)

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Investigators at the Northern Regional Research Laboratory of the U. S. Department of Agriculture have recently reported the isolation in pure state of a new antibiotic, hydroxystreptomycin. It is produced by an organism, isolated from Japanese soil, which they believe to be different from other streptomycin-producing strains. The new species has been designated as *Streptomyces griseocarneus* n. sp. On the basis of degradation experiments, Benedict *et al.*(1) have assumed that the new antibiotic differs from streptomycin only in having a hydroxy group in place of one of the hydrogens of the streptose methyl group. Accordingly, they have assigned the name "hydroxystreptomycin" to the new member of the streptomycin series.

One mg analytically pure hydroxystreptomycin trihydrochloride, when assayed against *Bacillus subtilis*, NRRL B-765 (ATCC 6633), was found to be equivalent to 784 μg of streptomycin base. The hydroxystreptomycin trihydrochloride used in these studies was prepared in the Northern Regional Research Laboratory and assayed 596 μg/mg (76% pure). The streptomycin sulfate assayed equivalent to 621 μg of pure free base per mg and was 78% pure.†

In the present studies we have compared the acute toxicity of hydroxystreptomycin trihydrochloride against the toxicity of streptomycin sulfate in white mice by subcutaneous administration. Both samples of streptomycin were dissolved in glass distilled water just before use, and the pH of each solution was measured with a glass electrode. The pH of a 10% solution of hydroxystreptomycin trihydrochloride was 7.1; that of a 10% solution of streptomycin sulfate was 4.8, which was adjusted to 6.2 with solid sodium bicarbonate. At least 10 mice (sexes equally divided) were used at each dosage level in determining the toxicity of the 2 streptomycins. For hydroxystreptomycin trihydrochloride, 46 mice were used and for streptomycin sulfate, 60 mice were used. The approximate LD_{50}, as determined by Trevan's method, was found to be as follows: for hydroxystreptomycin trihydrochloride, 865 mg/kg; and for streptomycin sulfate, 970 mg/kg.

The reactions observed in mice receiving either of the two streptomycins were essentially the same as those reported in the literature for streptomycin. With doses above the LD_{50}, death of mice usually occurred within 30 minutes after injection. Death was invariably due to respiratory paralysis; the heart continued to beat for some time after the respiration had ceased. At these elevated dosage levels some mice developed convulsions, particularly with doses of 1000 to 1200 mg/kg. With the lower range of dosage (400 to 800 mg/kg) most of the mice failed to show serious symptoms. For 30 days following administration, all surviving mice were observed daily for any local tissue reaction at site of injection and for delayed neurotoxic effects. In none of the mice was there any evidence of tissue reaction (irritation, necrosis), or neurotoxic symptoms (changes in gait and posture, ataxia, vestibular dysfunction).

From the above observations, it is concluded that hydroxystreptomycin trihydrochloride has about the same order of toxicity for mice by subcutaneous administration as streptomycin sulfate.

* Report of a study made under the Research and Marketing Act of 1946.

1. Benedict, R. G., Stodola, F. H., Shotwell, O. L., Borud, A. M., and Lindenfelser, L. A., *Science*, 1950, v112, 77.

† Data supplied by Fermentation Division, Northern Regional Research Laboratory, Peoria, Illinois.

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Hyaluronic Acid Utilization by Hemolytic Streptococci in Relation to Possible Hyaluronidase Function in Pathogenesis. (18526)

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Various polysaccharides have been shown to form the prosthetic groups of biologically-active proteins. In consequence, it is frequently the specific immune response to a particular polysaccharide which constitutes a major body defense to an infectious disease agent. In the main, attention has been focused on the polysaccharide as a functional unit. Little consideration has been given to the possibility that polysaccharides, *per se*, and their degradation products may also function in pathogenesis as a source of energy for disease-producing micro-organisms. Our concern in this paper is to consider this possibility in hemolytic streptococcal infections by utilizing the naturally-occurring polysaccharide, hyaluronic acid, which is the principal intercellular cementing substance of tissues derived from the mesenchyme. Upon hydrolysis, hyaluronic acid yields equimolar fractions of N-acetylglucosamine and glucuronic acid.

Material and methods. In our investigation of the effect of hyaluronic acid and its hydrolytic products upon the respiration of group A streptococci, we separated the latter into two groups: group I, which included 3 streptococcal strains not producing hyaluronidase in neopeptone, beef heart infusion broth and group II, containing 4 strains which did produce the enzyme in this medium. As we have reported elsewhere(1), the group II organisms have been found to produce higher percentage mortalities in chick embryos than group I organisms. All cell suspensions were washed once in phosphate buffer and standardized turbidimetrically using an Evelyn colorimeter. Oxygen uptake was measured in the Warburg apparatus in the usual fashion using a pH 7.4 phosphate buffer and 0.2 ml of a 20% KOH solution in the center well for CO_2 absorption. Oxygen uptake figures when reported by group represent combined averages for the individual strains comprising the group. Hyaluronidase activity was assayed by the turbidimetric method of Kass and Seastone as modified by Meyer(2). Purified hyaluronic acid was prepared from umbilical cords by the method of McClean as modified by Rogers(3). Hyaluronidase was prepared by ammonium sulphate fractionation of a culture of a group A type 4 streptococcus grown in dextrose, neopeptone beef heart infusion broth.

Results. In Fig. 1 it can be seen that groups I and II streptococci had low endogenous respirations. With hyaluronate as substrate, group I respiration increased an average 2.5-fold whereas group II organisms showed an oxygen uptake 5.2 times greater than the endogenous. The respiration of a virulent (for chick embryos) group II streptococcal strain (No. 421) was then measured in the presence of hyaluronate and with

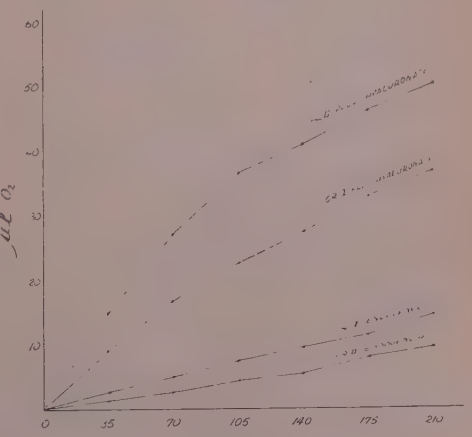

Fig. 1.

Hyaluronate stimulation of respiration in hyaluronidase-nonproducing and producing strains.

1. Sallman, B., and Birkeland, J. M., *Ann. N. Y. Acad. Sci.*, 1950, v52, 1062.

2. Meyer, K., *Physiol. Rev.*, 1947, v27, 335.

3. Rogers, H. J., *Biochem. J.*, 1945, v39, 435.

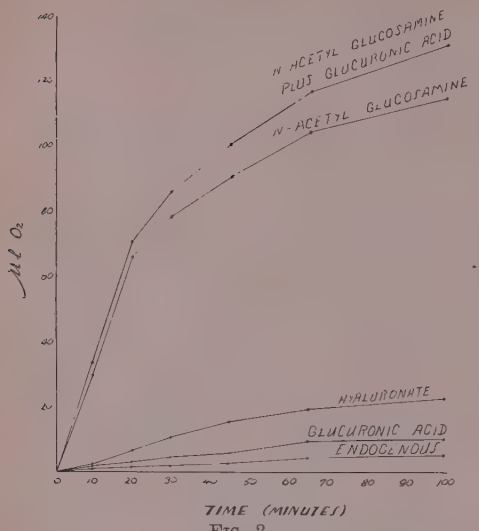

FIG. 2.
Stimulation of respiration (strain 421) by hyaluronate and hyaluronate breakdown products.

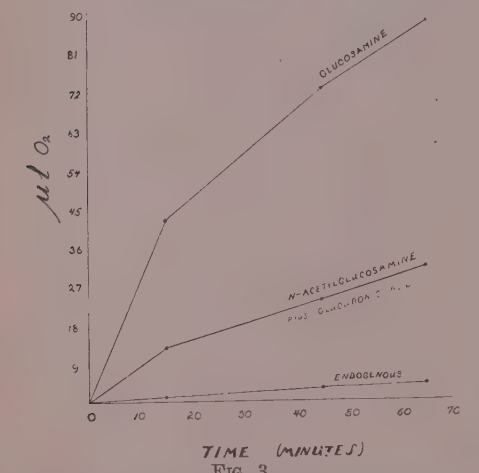

FIG. 3.
Stimulation of respiration (strain Sa) by hyaluronate hydrolytic products and glucosamine.

amounts of N-acetylglucosamine and glucuronic acid equivalent to their concentrations in the unhydrolyzed hyaluronate sample tested. The results in Fig. 2 show that in 100 minutes hyaluronate stimulated oxygen uptake 4-fold, N-acetylglucosamine 20-fold and glucuronic acid but slightly over the endogenous respiration. The addition of N-acetylglucosamine and glucuronic acid simultaneously resulted in an oxygen uptake equivalent approximately to the sum of their individual stimulations. This stepwise degradation of hyaluronate associated with a source of increased available energy suggested that the process might be continued with the deacetylated product, glucosamine. In Fig. 3 it can be seen that an equivalent amount of glucosamine stimulated respiration of another group II organism (strain Sa) to an extent 3 times as great as did N-acetylglucosamine plus glucuronic acid.

Since hemolytic streptococci are frequently found as co-invaders in the host with other pathogenic micro-organisms, the possible synergistic utilization by the latter of hyaluronic acid was investigated. A number of organisms frequently found associated with group A streptococci *in vivo* were tested with hyaluronate alone as a source of energy and with hyaluronate in the presence of streptococcal strain No. 421. From Table I it is evident that all of the organisms tested, with the exception of *N. catarrhalis*, *C. diphtheriae* and possibly *S. aureus*, utilized hyaluronate for respiration. In the presence of a group A streptococcus, each micro-organism without exception accomplished a more efficient utilization as demonstrated by the streptococcal: hyaluronate ratios ranging from 1.2 to 5.5 for the nonpathogens and 1.1 to 5.8 for the potential pathogens.

It is important to know whether an organism which utilizes hyaluronate for respiration attacks the molecule directly or whether enzymatic hydrolysis into the component N-acetylglucosamine and glucuronic acid, presumably through the agency of hyaluronidase, must occur prior to its utilization. From our findings, it appears that hyaluronate itself is not utilized but that the resultant oxygen uptake in the presence of hyaluronate can be accounted for by the products formed in its hydrolysis. The results of a typical experiment designed to answer this question are given in Table II. Here, it is seen that within 2 hours *E. coli* consumed 502 μl of oxygen with 0.5 mg of hyaluronate as a substrate. When 165 Turbidity-Reducing Units of hyaluronidase were added, respiration was increased to 574 μl. When a similar

TABLE I. Aerobic Utilization of Hyaluronate by Micro-organisms Frequently Found Associated with Group A Streptococci.

Organism	Endogenous respiration	Hyaluronate	Group A streptococcal synergism*	Strep.: hyaluronate ratio
	Nonpathogens			
E. coli 'R' communior	77†	383	460	1.2
N. catarrhalis	17	18	61	3.4
B. subtilis PCI 220	68	131	721	5.5
	Potential pathogens			
D. pneumoniae, type I, Abbott	60	76	82	1.1
S. aureus 209-P	70	77	123	1.6
M. tuberculosis H 37 Ra‡	66	413	828	2.0
C. diphtheriae	99	101	307	3.0
H. influenzae H-2040	57	96	406	4.2
H. pertussis	70	84	490	5.8

* Corrected for streptococcal respiration in the presence of hyaluronate.
† μl O_2.
‡ Eighteen-day growth in Dubos' basal medium without Tween 80.

TABLE II. Mechanism of Hyaluronate Utilization in the Respiration of *E. coli*.

	μl O_2 consumed in 2 hours	
Endogenous	107	
Hyaluronate (.5 mg)	502	
" (.5 mg) + hyaluronidase (165 TRU*)	574	
Hyaluronate (.5 mg) + hyaluronidase (165 TRU) previously incubated 37°C 45 min.	577	
N. acetylglucosamine (.266 mg)	302	572
Glucuronic acid (.234 mg)	270	
Hyaluronidase:hyaluronate ratio	1.14	

* Turbidity-reducing units of the streptococcal enzyme.

amount of hyaluronate was incubated with hyaluronidase prior to being added to the Warburg flask, an equivalent amount of oxygen was taken up (577 μl). Amounts of N-acetylglucosamine (0.266 mg) and glucuronic acid (0.234 mg) equal to their concentration in the unhydrolyzed hyaluronate yielded a total oxygen consumption of 572 μl. The disparity between oxygen uptake with hyaluronate (502 μl) and hyaluronate plus hyaluronidase (574 μl) can be explained by assuming that hyaluronidase production by the organism was insufficient for complete hydrolysis of the substrate. The likelihood of this explanation is enhanced by the fact that the addition of 10 TRU in place of the 165 TRU given in Table II will accomplish the same oxygen uptake.

Evidence that hyaluronidase action is also responsible for the synergistic utilization of hyaluronate may be gleaned from the fact that the streptococcal:hyaluronate ratio of the *E. coli*-group A streptococcus complex (Table I) is 1.2, essentially the same as the hyaluronidase:hyaluronate ratio for *E. coli, viz,* 1.14 (Table II). Similarly, the streptococcal:hyaluronate ratio of the *M. tuberculosis*-group A streptococcus complex is 2.0 compared to a hyaluronidase:hyaluronate ratio of 2.2.

Discussion. When the culture supernatants of 7 of the organisms listed in Table I (*H. influenzae* and *H. pertussis* were grown on solid media) were assayed for hyaluronidase by the turbidimetric and mucin-clot-prevention methods, no enzyme was found. Four of these organisms, however, were able to utilize hyaluronate, apparently producing the enzyme adaptively in the presence of the specific substrate. This behavior cannot be considered anomalous since we have observed repeatedly that many strains of hemolytic streptococci, which do not produce hyaluronidase in the usual culture media, do produce the enzyme after one of more serial transfers in media containing hyaluronate. This fact would indicate that previous surveys(4) of organisms, grown in media free of hyaluronate, for hyaluronidase production should be revaluated since the substrate is available during infection in the host. Another point which should be mentioned at this time is the relative in-

4. Crowley, N., *J. Path. Bact.*, 1944, v56, 27.

sensitivity of the turbidimetric and MCP tests for hyaluronidase assay. Small amounts of hyaluronidase (considerably less than 1 TRU/ml) could be detected manometrically when included with hyaluronate and a suitable micro-organism in the Warburg flask but could not be measured by the turbidimetric and MCP methods. The failure of the latter procedures was also evident in our inability to demonstrate hyaluronidase titers in Warburg vessels during active hyaluronate utilization by the various bacterial organisms.

Apparently, when hyaluronate is present as a substrate, group I streptococci, not producing hyaluronidase in infusion broth, do produce the enzyme in an adaptive fashion. The extent of such adaptive enzyme formation, however, is less than the amount of enzyme normally produced by group II streptococci. This is evident from the fact that twice as great a stimulation of respiration occurred with the group II organisms, indicating that the latter streptococci made greater amounts of N-acetylglucosamine and glucuronic acid available as sources of energy for the respiring cells. The wide distribution of hyaluronic acid in the body may serve as an energy reservoir for bacterial invaders possessing hyaluronidase in their metabolic repertoire. Furthermore, in the presence of such an invader, it is probable that other micro-organisms producing disease may benefit from the degradation products, N-acetylglucosamine and glucosamine, so produced. In this connection it may be well to recall to mind how frequently hemolytic streptococci are found as invaders causing primary, concurrent or secondary diseases. Such associations might be considered synergistic, the streptococci providing a ready source of energy and receiving in return a debilitated body defense due to the activities of the associated organism.

In evaluating the likelihood that just such a state of affairs might exist in the invaded host, let us consider briefly the fate of N-acetylglucosamine in the body. Here, N-acetylglucosamine is deacetylated only with difficulty if at all(5). Deamination is accomplished by kidney, testis, brain and certain muscle tissues only under aerobic conditions. The liver contains no enzymes able to oxidize or deaminate the amine sugars. Thus, N-acetylglucosamine produced and liberated in the body will not be removed or acted upon very readily. This represents an ideal situation for its utilization by invading bacteria. In contrast, many bacteria are able to deacetylate and deaminate N-acetylglucosamine as well as oxidizing both it and glucosamine rather rapidly under anaerobic as well as aerobic conditions.

From the clinical point of view, information is slight but still suggestive. In various infections, the adult blood glucosamine levels have been found to exceed by far the normal range of 76 to 110 mg %. The highest glucosamine titers have been observed in cases of rheumatic fever, rheumatoid arthritis, hemolytic streptococcal pharyngitis, pneumococcal pneumonia, staphylococcal infection and tuberculosis(6). All of the organisms presumably causing these diseases have been shown to be able to utilize hyaluronate and, in many cases, to produce appreciable amounts of hyaluronidase.

Hitherto, the possible role of hyaluronidase in pathogenesis has been visualized solely as a means of increasing the local tissue invasiveness of hyaluronidase-producing micro-organisms. We are of the opinion that further investigation of the role of hyaluronidase in the respiration of hyaluronidase-producing organisms and of the function of hyaluronic acid hydrolytic products in the metabolism of associated agents of infection will yield a more comprehensive picture of a mechanism of disease production.

Summary. Hemolytic streptococcal strains, virulent for chick embryos, were found able to utilize hyaluronic acid for respiration to a greater extent than relatively avirulent strains. With the micro-organisms tested, it appears that the hydrolytic products and not hyaluronate itself are utilized in respiration, such hydrolysis occurring presumably through the agency of hyaluronidase. Pathogenic bacteria frequently found associated with group

5. Lutwak-Mann, C., *Biochem. J.*, 1941, v35, 610.

6. West, R., Clarke, D. H., and Kennedy, E. M., *J. Clin. Invest.*, 1938, v17, 173.

A streptococci *in vivo* had a synergistic relationship with the latter in utilizing hyaluronate as a substrate for energy. On the basis of these findings, a mechanism of pathogenesis by hemolytic streptococci alone and with associated agents of infection was proposed. Certain clinical findings, interpreted as evidence for the existence of such a process, were cited.

It is a pleasure to acknowledge the assistance of Miss Clara Boyd.

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A New Dialyzer for Use as an Artificial Kidney. (18527)

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In the wake of pioneer experimental studies of Abel, Rowntree and Turner(1), the process of dialysis is receiving steadily increasing attention in the treatments of patients with acute anuria(2-8), and in the study of hypertension and renal disease(9,10). Our special interest has been in the application of continuous extracorporeal dialysis of blood for the correction of disturbed ionic equilibrium and the removal of accumulated nitrogenous constituents of the body water as occurs in uremic states. The instrument that was designed incorporates the following desirable features: (1) sterilization by autoclaving, (2) complete visibility of blood and wash channels during operation, (3) provision for rapid exclusion and short-circuiting of defective tubing, (4) true countercurrent flow, (5) surface area adaptable to needs, (6) relative constancy of blood volume, (7) optional and controlled pressure differential between blood and wash electrolyte solution compartments, and (8) reasonable compactness.

A complete dialyzer* is made up of a variable number of units according to the surface area desired, an assembled unit being shown in Fig. 1. Each unit has a cellophane surface area of approximately 4000 cm^2. Fig. 2 and 3 are diagrammatic representations of the top and cross sectional views of such a unit. Top and bottom are formed from a heavy, thermal stress-resistant sheet of plate glass (A), the steel frame (B) is in the middle, and clear plastic gasket sheets (I) are placed between glass and steel. As seen in Fig. 1 the steel frame of each unit forms two double channels, each about one meter in length. After preparation of the cellophane tubing (Visking Corp., 36/32 in. inflated diameter) by repeated boiling in water, and testing with air pressure for leaks, a length of tubing (D) is connected to the nylon fittings (E), sandwiched between the flexible steel, woven, chain-linked screens (C) and placed into a channel. The nylon fittings (cross section Fig. 2) are constructed to provide smooth flow without dead space, folding or leakage. Attachment is made by drawing the end of the cellophane tubing over the inner nylon piece and slipping the steel collar on top. The nylon fittings are then inserted into the openings at both ends of the channel. Turn-

1. Abel, J. J., Rowntree, L. G., and Turner, B. B., *J. Pharm. and Exp. Therap.*, 1914, v5, 275.
2. Kolff, W. J., *J. Mt. Sinai Hospital*, 1947, v14, 71.
3. Murray, G., Delorme, E., and Thomas, N., *Arch. Surg.*, 1947, v55, 505.
4. Alwall, N., *Acta med. Scand.*, 1947, v128, 317.
5. Skeggs, L. T., and Leonards, J. R., *Science*, 1948, v108, 212.
6. Snapper, I., *Med. Clin. N. Am.*, 1950, v34, 509.
7. Merrill, J. P., Thorn, G. W., Walter, G. W., Callahan, E. J., and Smith, L. H., *J. Clin. Invest.*, 1950, v29, 412.
8. Darmady, E. M., *Proc. Royal Soc. Med.*, 1948, v41, 418.
9. Vanatta, J., Muirhead, E. E., and Grollman, A., *Am. J. Physiol.*, 1949, v156, 443.
10. Grollman, A., Muirhead, E. E., and Vanatta, J., *Am. J. Physiol.*, 1949, v157, 21.

* Made by Brosites Machine Co., New York City.

Fig. 1.
Assembled dialyzer unit showing 4 channels.

ing a nut (H) on the outside of the apparatus seals the cellophane-nylon junctions by compressing the collar over the inner piece. The sturdy screens (C), placed on both sides of the cellophane tubing, compress it to about 1 mm thickness. The volume of the blood inside the tubing thereby depends only on the elasticity of the cellophane with changes in pressure and is constant at constant pressure (the volume at a differential pressure of 50 mm Hg is about 300 cc per unit). The connections to the nylon fittings of adjacent channels are made externally with short segments of Tygon tubing. The wash solution flows along the cellophane tubing, though opposite in direction to the blood, to the end of a channel, passes through an opening in the steel frame and enters the adjacent channel. The wash is drained off from the top through a metal tube drain (F). The highest efficiency of transfer is established by a true counter-current flow without channeling. A concentration gradient that is constant, with an optimal rate of exchange, is thereby established. This principle has been extensively used in various commercial dialyzers for laboratory and technological purposes and was first incorporated by our group for clinical use(18). The unit becomes completely sealed upon the tightening of bolts (J) extending through fibre washers in top and bottom plates. This instrument has been reassembled

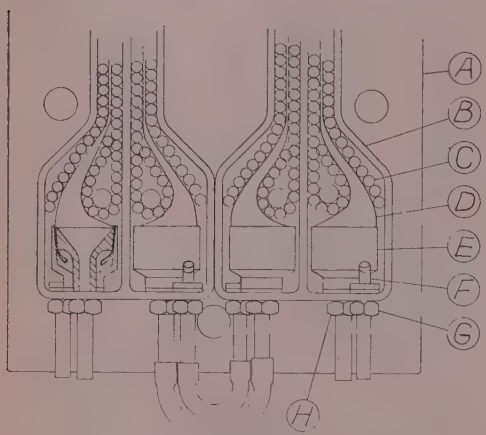

Fig. 2.
Top view of the front end of the dialyzer.

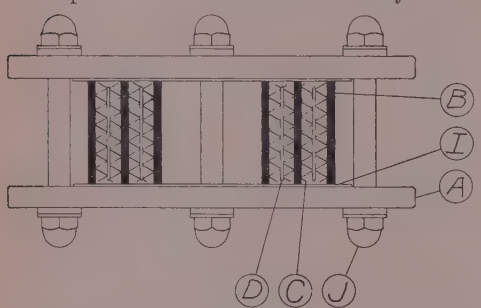

Fig. 3.
Cross-sectional view at the middle of the dialyzer.

18. Rosenak, S. S., Oppenheimer, G. D., Saltzman, A., Exhibit at the annual meeting of the Amer. Urol. Soc., Boston, 1948.

many times, with and without autoclaving, and we found it sufficient to achieve a snug fit by tightening the bolts with an ordinary wrench without danger of breakage of the glass plates. After assembly, and testing for leaks by placing water in the wash compartment and air in the cellophane under pressure of 120 mm Hg, the unit is drained off, nuts are loosened to permit expansion and the unit autoclaved as a whole. Immediately thereafter, while the membrane is still damp, all fittings are tightened, the connecting tubing applied to corresponding fittings and the inner compartment filled with sterile saline. Should the instrument be sterilized for possible use within a time period of about three weeks, instead of filling the units with saline, all outside blood channel connections are joined with each other by means of autoclaved Tygon tubings, while the wash channel connections are similarly joined with autoclaved rubber tubings. The dialyzer thus sealed will maintain sterility for at least 30 days as tested by us repeatedly. The sealed-in moisture prevents the dialyzing membrane from shrinking and getting brittle.

All connections to the dialyzer can be handled from the outside without breaking sterility. Complete visibility of operation is assured by the clear top and bottom plates, clear gaskets and solutions. Any subsequent leakage of blood, which may occur, is immediately detected, located and by-passed. Constant temperature is assured by slow dissipation of heat from the instrument, by warming the wash solution prior to entry into the dialyzer, and is checked by thermometers inserted into channels (Fig. 1). For the sterile propulsion of materials to the dialyzer at appropriate rates, a suitable pump of new design, previously described(15), has been used. In operation our pump permits conclusions (by pre-calibration) as to the speed of blood flow through the dialyzer, this being the resultant of 2 components: the setting of the amount of compression of the tubing and the operation pressure as recorded by a manometer at the blood inlet. The latter is attached to a bubble-catcher which also acts as a sampler.

The resistance of the dialyzer itself allows fast rates of blood flow without undue elevation of intraluminal pressure as can be seen from the following. In recirculation *in vitro* arrangement with the blood passing through a circuit consisting of a reservoir (open glass receptacle), the pump, an inflow bubble catcher, a 12 channel dialyzer hooked up in series, outflow bubble catcher, and return to the reservoir, a blood flow of 500 cc/min was obtained with a pressure of 45 mm Hg inside the cellophane tubing. In *in vivo* operation we similarly find that the intraluminal pressure is more dependent on the resistance created by the catheters leading to and from the venous compartments of the patient, than on the dialyzer. If ultrafiltration is desired a small adjustable clamp is placed on the return side of the blood flow to elevate the resistance of the system to suitable levels. Such controlled hydrostatic pressure is of considerable importance as it permits lowering of the glucose concentration in the electrolyte rinsing solution to more physiological levels, while preventing the exchange of water molecules between the electrolyte rinsing and blood compartments(16). With our apparatus the calculated rate of ultrafiltration at 37°C, an average pressure differential of 105 mm Hg and a cellophane thickness of 0.0008 in., was 0.02 cc/cm^2 of surface area/hour.

A rather important feature of the described apparatus is that it can be sterilized by autoclaving after being fully assembled. Following the latter step the entire unit can be stored in the sterile state, thereby being available for instant use. The autoclaving process adds a margin of safety over instrument sterilized by other means rendering the dialyzer safe for use even after a run on a patient with infectious hepatitis.

Resistance to blood flow is low in this instrument in spite of a good surface to volume ratio obtained by restraining the membrane to 1 mm thickness. It is thus possible to connect the blood compartments in series

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rather than parallel up to a surface area of about 10,000 cm^2 with complete avoidance of channeling which is a necessary drawback to any parallel arrangement without adequate flow control. The dialyzers of MacNeill(11) and of Skeggs and Leonards(12) which are characterized by parallel arrangement of numerous dialyzing compartments show various degrees of channeling. The blood flow in the individual compartments is necessarily uneven (13) and cannot be remedied by stepping up the rate of blood flow. Thus, the blood remaining for longer periods in the "slow" compartments may develop a clotting tendency which in turn leads to the admixture of microscopic blood clots or fibrin strands(14) to the circulating blood. The difference in the resistance of the above dialyzers and the one to be described can further be seen from data as to intraluminal pressures during blood flows of 200 to 300 cc/min. With our design the pressure is 40 to 60 mm Hg, the Skeggs and Leonards instrument runs at 170 to 180 mm Hg, at which a considerable amount of ultrafiltration is produced requiring the addition of parenteral fluids to the patient. With our instrument the pressure inside the cellophane, and thereby the degree of ultrafiltration, can be optionally regulated by the application of an external resistance.

A further advantage of our design is in the use of a chain-linked screen, made of stainless steel, for compression of the membrane. This accomplishes a minimal reduction in surface area of the membrane while providing rigidity in prevention of membrane expansion, along with ease in preparation and removal. Finally, the complete visibility of the blood channels during operation permits location and remedying of defective channels (by short-circuiting) without breaking sterility nor interruption of operations for more than one minute. This is impossible in the multilayer design in which a mishap calls for complete disassemblage. Dialysis of an aqueous solution of creatinine in various concentrations was performed with our instrument against water at 37°C with 3500 cm^2 of cellophane in the dialyzer. The creatinine solution flowed at a rate of 130 cc/min, and tap water in counter current at 100 cc/min. The rate of dialysis of creatinine under these conditions is given in Table I.

TABLE I. Dialysis of Creatinine at Various Concentrations.

Creatinine level, mg %	Creatinine dialyzed/hr, mg
5.6	75
7.8	101
10	166
20	335

Under the above conditions of temperature, flow rates, and surface area, with 200 mg % urea solution, a dialysis of 5 g urea per hour was effected (urea clearance of 42 cc/min). This compares favorably with the clearance of the "Artificial Kidney" of Skeggs *et al.*(12) which is 50-80 cc/min/12,000 cc surface area. Such surface area can be obtained by using 3 of our units in parallel flow. Such a battery could yield easily a urea clearance of 125 cc/min. Similarly a 5% ammonium sulfate solution was dialyzed against water over a surface area of 7500 cm^2. The sulfate solution flowed at a rate of 15 cc/min while the speed of the tap water was 400 cc/min at a temperature of 41°C. Under these conditions the concentration of the sulfate solution dropped during the run to 0.02%. Surface areas of 4000 to 12,000 cm^2 have been applied to the dialysis of uremic patients(17). To illustrate the effectiveness of this instrument we quote an experimental case of terminal uremia in a patient with chronic glomerulonephritis. In a 3 hour run on this patient with an initial blood urea nitrogen of 230 mg %, 50 g of urea nitrogen was cleared over a cellophane area of 9500 cm^2, at an average blood speed of 300 cc/min and an average electrolyte "wash" flow rate of 200 cc/min. 8 hours after conclusion of dialysis

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the blood urea nitrogen dropped to 150 mg %.

The electrolyte solution was prepared from sterile concentrates† and had the following standard composition as expressed in g %. Dextrose 1, NaCl .610, $NaHCO_3$ .220, KCl .0360, $CaCl_2$. $6H_2O$ .0450, $MgCl_2$. $6H_2O$ .0107, $NaH_2PO_4H_2O$ .0058.

Summary. An efficient and safe dialyzer‡ suitable for continuous operation under sterile conditions is described. It is made up of a variable number of component channels according to surface area desired. Sterility is accomplished by autoclaving as a whole and optional controlled ultrafiltration is possible.

† Obtained by courtesy of Eli Lilly Co., Indianapolis.

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Chemotherapeutic Effectiveness of Alloxan in Murine Bartonellosis.* (18528)

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Alloxan, an oxidation product of uric acid, is present in combined form in several compounds which are of importance in mammalian physiology, and there is some evidence for its occurrence in the free state in blood and other animal tissues(1). It has been extensively studied in recent years because of its specific toxic action on the beta cells of the pancreatic islands, resulting, when suitable dosages are used, in the experimental production of diabetes(2,3). There is evidence that its diabetogenic effect is caused by reaction with SH groups in enzyme systems(4). Alloxan, as far as we can learn, has not previously been tested for chemotherapeutic activity in any infectious disease.

Material and methods. Latently infected (carrier) rats of the St. Louis University strain were used. Previous work with several hundred rats of this strain has shown that splenectomy is invariably followed by severe bartonellosis. The organisms appear in the erythrocytes on the 2nd or 3rd day after removal of the spleen (occasionally on the 1st day), reach a maximum on the 5th or 6th day, and disappear, in surviving animals, by the 9th or 10th day. The highest daily bartonella counts are rarely below 100 per 100 erythrocytes, and the mortality is usually about 50%.

All rats in the experiments to be reported here were maintained throughout the observation periods on a low sulfur diet (LSD) with the following composition: casein 6%, salt mixture 4%, sucrose 15%, corn starch 48%, lard 22%, cod liver oil 3%, and debitterized brewers yeast powder 2%.

In Exp. 1, 20 rats, previously maintained for 10 days on LSD, were splenectomized. Ten of these served as controls. The remaining 10 were given daily intraperitoneal injections (40 mg per kilo) of freshly prepared solutions of alloxan in distilled water for 13 days, starting 4 days before splenectomy.

In Exp. 2, 14 rats, previously maintained for 3 days on LSD, were splenectomized. Four rats served as controls. The remaining 10 were given daily intraperitoneal injections

* This investigation was supported, in part, by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

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TABLE I. (Exp. 1). Effect of Alloxan on Murine Bartonellosis in Splenectomized Carrier Rats.

Rat No.	Treatment	Lowest hemoglobin level, mg	Highest bartonella count (per 100 rbc)	Outcome
1	Control	5.5	252	S*
2	,,	4.5	214	,,
3	,,	5.0	293	,,
4	,,	4.0	188	,,
5	,,	4.0	90	,,
6	,,	6.0	273	Died 6th day
7	,,	5.5	315	S
8	,,	5.5	252	,,
9	,,	6.0	218	,,
10	,,	5.5	162	Died 6th day
11	Alloxan	16.5	0	S
12	40 mg/kg	16.5	0	,,
13	daily	17.0	0	,,
14	,,	17.0	0	,,
15	,,	16.5	0	,,
16	,,	16.5	0	Died 5th day†
17	,,	15.5	0	S
18	,,	16.0	0	,,
19	,,	16.5	0	,,
20	,,	16.5	0	,,

* Survived.
† Splenectomy wound broken down. No hematological evidence of bartonellosis.

TABLE II. (Exp. 2). Effect of Alloxan on Murine Bartonellosis in Splenectomized Carrier Rats.

Rat No.	Dosage	Treatment started	Highest bartonella count	Outcome
1	—	Control	182	S
2	—	,,	299	Died 6th day
3	—	,,	249	,,
4	—	,,	301	,,
5	40 mg/kg	1 day p.s.*	0	S
6	,,	1 day p.s.	0	,,
7	,,	day of spl.	0	,,
8	,,	day of spl.	0	,,
9	,,	1 day a.s.†	0	,,
10	,,	1 day a.s.	0	,,
11	,,	2 days a.s.	0	,,
12	,,	2 days a.s.	0	,,
13	,,	3 days a.s.	0	,,
14	,,	3 days a.s.	0	,,

* p.s. = After splenectomy.
† a.s. = Before splenectomy.

of alloxan at various intervals before the expected appearance of organisms in the blood. Treatment was stopped on the 8th day after splenectomy.

In Exp. 1, the effectiveness of the agent was judged by counting the number of organisms in giemsa-stained blood films, and by hemoglobin determinations. These observations were made daily for 12 days, starting on the day after splenectomy. The bartonella counts were based on the examination of 400 erythrocytes, and were recorded as organisms per 100 erythrocytes. Hemoglobin was determined directly by the Dare method. In Exp. 2, bartonella counts only were made, and the observation period was 14 days.

Results. In Exp. 1 (Table I) the 10 controls showed the expected severe anemia and parasitization of erythrocytes, while the rats given alloxan showed no evidence of anemia or bartonella infection.

The results of Exp. 2 (Table II) again showed complete suppression of the infection by daily injections of 40 mg per kg of alloxan, even when started the day after splenectomy, when multiplication of the organisms had probably already begun.

The animals surviving in these experiments, as well as in other similar experiments, were observed daily for several weeks after bartonella counts were discontinued, and no clinical evidence of delayed bartonellosis was noted. It seems probable, though not yet proved, that rats given alloxan at this dosage level were rendered bartonella-free. In another series of rats, not included in the tables, daily injections of 20 mg per kg for 12 days completely suppressed the infection, but typical bartonellosis occurred in several animals 4 to 5 days after cessation of therapy.

Discussion. The diabetogenic action of alloxan may be inhibited by BAL, glutathione, cysteine, and related compounds containing the SH group, but only if the protective substance is given almost simultaneously with the alloxan. For this reason, it has been assumed that the pancreatic injury results from inactivation of thiol groups(4). It will be of interest to determine whether the therapeutic action of alloxan can be neutralized by the compounds mentioned above.

The dosage of alloxan used in these experiments is well below that required for the production of diabetes. It has been shown, however, that a diminution in glucose toler-

ance is caused in rats by the intravenous injection of as little as 25 mg per kg every other day for 4 weeks, although blood sugar levels remain normal while the rats are on a normal diet(5).

Inhibition of bartonella multiplication is effected physiologically by the presence of the intact spleen, and artificially by arsenicals(6), by aureomycin and terramycin(7), and by alloxan. Arsenicals and alloxan are known to inactivate thiol groups. In spite of extensive study, the mechanism by which the spleen inhibits the growth of *Haemobartonella muris* has not been discovered(8).

In future work, the possibility that alloxan may have chemotherapeutic activity in infections other than bartonellosis will be explored, and a variety of closely related compounds will be tested. The observation reported here suggests the possibility that compounds formed naturally in the course of nucleic acid metabolism may be chemotherapeutically effective.

Conclusions. Alloxan in relatively low and presumably nondiabetogenic dosages was found to be effective in preventing the development of murine bartonellosis, which invariably follows splenectomy in untreated carrier rats. The activity of this agent probably depends on its reaction with SH groups.

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Factors Influencing Effect of Radioactive Colloidal Gold on Free Tumor Cells in Peritoneal Fluid.* (18529)

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In a previous note(1) a method has been outlined for screening quantitatively the effect of radioactive isotopes on tumor cells in mice: cells of Sarcoma 37 were grown serially as free cells in the peritoneal fluid of CFW mice and tested for their resistance to various doses of a radioactive isotope injected intraperitoneally. Using this technic in experiments with radioactive colloidal gold(2) it has been found that sarcoma cells grown in the peritoneal fluid of a CFW mouse 2 to 5 days after inoculation of 10,000 cells, disintegrated and completely disappeared within 48 hours after i.p injection of 0.3 to 0.42 mc of radioactive colloidal gold. In a new series of experiments with several groups of mice, we have analysed this radiotherapeutic effect† by varying in each group of mice one of the conditions of the experiment.

Material and methods. Two strains of tumors were used in this work: (1) Sarcoma 37 was grown in 2 strains of mice—CFW and dba; (2) malignant lymphoma (isolated originally from the thymus of a dba mouse) in dba mice. Thus, dba mice were used for carrying 2 different tumors. Both tumors were propagated in serial transfers in the peritoneal fluid. The technic of inoculation with a requisite number of tumor cells and the method for estimating the therapeutic results

* This work was carried out under Contract At-(40-1)269 with the Division of Biology and Medicine, U. S. Atomic Energy Commission.

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2. Goldie, H., and Hahn, P. F., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 638.

† Colloidal radioactive gold containing Au^{198} was obtained from Abbott Laboratories, North Chicago, Ill.

3. Goldie, H., and Felix, M. D., *Cancer Research,* 1951, v11, 73.

TABLE I. Effect of Radioactive Colloidal Gold on Free Tumor Cells in the Peritoneal Fluid of the Mouse Under Various Experimental Conditions.

Dose of RCG, mc	Route	Strain of tumor	Strain of mice	Cells inoculated, No.	Days between inoculation and treatment	Inoculated mice surviving after treatment with			Inoculated mice showing disappearance of tumor cells in perit. fluid after treatment with		
						RCG alone, No.	RCG and NCG, No.	Controls, No.	RCG alone, No.	RCG and NCG, No.	Controls, No.
.3	I.p.	S-37	CFW	100000 to	5 or 6	63/2	48/17	12/53	63/2	41/24	0/65
	I.v.			1000000		36/4	33/7	12/53	32/8	33/7	
.1						38/27	32/33	12/53	21/34	24/31	0/40
						14/26	18/22	12/53	12/28	16/24	
.05						14/26	12/28	8/32	12/28	10/30	1/39
						5/5	4/6	1/9	2/8	1/9	
.3			dba			23/32	13/42	7/23	21/34	13/42	3/27
						11/29	8/22	7/23	9/31	16/34	
.1						15/35	10/40	5/25	15/35	9/41	2/38
						18/32	22/28	5/25	16/24	12/28	
.05						8/12	8/12	6/14	2/18	2/18	1/19
						9/11	6/14	6/14	3/17	1/19	
.3		Malignant lymphoma		100000	7	20/0	18/2	4/16	20/0	18/2	2/18
						20/0	20/0		19/1	20/1	
.1						18/2	16/4	4/16	15/5	13/7	1/19
						13/7	15/5		13/7	12/8	
.05						10/10	8/12	5/15	10/10	8/12	
						7/13	8/12		7/13	7/13	
.3	I.p.				14	8/12	10/10	2/18	8/12	9/11	0/20
.1						5/15	7/13		2/18	7/13	
.05						2/18	2/18		1/19	2/18	
.3				1000000	7	5/15	4/16	3/17	4/16	2/18	0/20
.1						4/16	5/15	3/17	2/18	1/19	
.05						2/18	1/19	3/17	0/20	1/19	
.25 RCG stored in macrophages		S-37	CFW	10000	5	9/1		4/6	4/6		0/10
.1 as above						5/5		3/7	2/8		0/10

All results were checked 5 or 6 days after treatment.c colloidal gold.
RCG = Radioactive colloidal gold. NCG = Inactiv

were described elsewhere(1,3). Since it was found previously(2) that intraperitoneally injected colloidal gold (either radioactive or inactive) is condensed and stored by numerous macrophages of the peritoneal fluid, it was considered necessary to study the influence of the storage of radioactive gold in macrophages on tumor cells. For this purpose, the amount of radioactivity in a suspension of macrophages containing gold (separated from serous fluid by centrifugation) was determined in a Geiger counter (using as standard for comparison an aliquot of the original preparation), and doses of suspension containing 0.1 or 0.25 mc were injected i.p into 20 mice previously inoculated with S-37 cells. Furthermore, it seemed reasonable to assume that the preparative intraperitoneal injection of a massive dose of inactive (decayed) colloidal gold (designated below as NCG) would modify the conditions for distribution of subsequently injected radioactive colloidal gold (designated below as RCG) in the peritoneal fluid, and therefore, its effect on peritoneal free tumor cells. For this reason, doses of 0.1 or 0.2 cc of inactive (decayed) colloidal gold (NCG) were injected i.p into inoculated mice 1 or 2 days

before their treatment with RCG, while other groups of similarly inoculated mice were treated with RCG alone, and controls remained untreated.

Results. Table I given below reviews results in experimental animals, *i.e.*, bearing tumor cells in the peritoneal fluid and treated with RCG. Only those findings are recorded in the table where the change in a condition of the experiment resulted in the change of the result. The conditions are indicated in the left side column; the therapeutic result is estimated by survival of treated mice (as compared with controls) and by disappearance of tumor cells from their peritoneal fluid. For convenience of comparison these results are shown in the same column for 3 series of mice, treated with RCG only, with NCG and RCG successively and untreated controls.

The data of Table I revealed the following variations in the radiotherapeutic effect on tumors: (1) The intensity of the effect was distinctly proportionate to the dose of injected RCG. The administration of 0.3 mc was responsible for disappearance of tumor cells from the peritoneal fluid and for extension of the life span in a significant proportion of treated mice (nearly all CFW with S-37 and dba inoculated with 100,000 lymphoma cells), while the dose of 0.1 mc produced this effect only in a relatively small number of mice of both strains and the dose of 0.05 mc only in a few mice with S-37. (2) Intravenous administration of RCG was less efficient than intraperitoneal treatment, but this difference was not striking. This finding is in agreement with our repeated observation that after treatment with 0.3 mc by either route the first marked signs of tumor cell disintegration (swelling, distorted mitoses) in the peritoneal fluid appeared consistently at the same time, *i.e.*, after about 4 hours. (3) For the same strain of tumor S-37, and the same procedure of inoculation and treatment, the effect in CFW mice was strikingly better than in dba mice. However, it was noted that dba mice showed higher percentage of subcutaneous tumors at the site of inoculation. (4) The effect on sarcoma cells was independent of the size of the inoculum within wide limits (10,000‡ to 1,000,000 cells) and to a certain extent (2 to 5 days) from the length of the interval between inoculation and treatment. However both factors modified significantly the effect on lymphoma, when the inoculum varied from 100,000 to 1,000,000 cells and the interval from 7 to 14 days. (5) The pre-treatment with NCG decreased significantly the effect of therapeutic doses (0.3 and 0.1 mc) of RCG on sarcoma in CFW mice and even more strikingly in dba mice, but not on lymphoma in dba mice. (6) The action of RCG stored in macrophages on sarcoma cells in peritoneal fluid appeared delayed.

Discussion. The intrinsic tendency of Sarcoma 37 cells to regression after initial multiplication in the peritoneal fluid(3) was a factor limiting the number and the vitality of tumor cells and thus contributing to the similar effect of the radiation, while this factor was absent in the treatment of free lymphoma cells in the peritoneal fluid(3). Their unchecked proliferation accounts for decrease of their response to the radiotherapeutic effect in conditions increasing cell multiplication (large size of the inoculum, long interval between inoculation and treatment), while for S-37 in the same conditions the increased cell multiplication is automatically countered by spontaneous cell regression. Similar differences in growth potency of tumors may account for striking differences in the results of radiotherapy in various cases of human tumors.

Our control experiments indicated higher invasiveness of free S-37 cells for tissues of dba mice: this difference may account for lower level of the radiotherapeutic effect of RCG on free S-37 cells in dba mice. Even for different mice of the same strain, variations in this effect may result from different invasiveness of the same tumor cells for different mice. Thus, the variations of the radiotherapeutic effect are manifestations of various combination of the action of radiation with biological factors inherent in tumor strain, mouse strains or individual mice.

A similar combination of factors was obviously responsible for the increase of mor-

‡ The results with 10,000 and 50,000 sarcoma cells were reported in a previous paper(1).

tality in mice inoculated i.p with S-37 and treated successively with NCG and RCG. This phenomenon was obtained only by combination of the following 4 conditions: (1) tumor cell multiplication in the peritoneal fluid; no influence of pre-treatment with NCG was noted in RCG treated normal mice or mice with subcutaneous tumors; (2) strain of tumor—no influence of NCG on the effect of RCG in mice inoculated i.p with malignant lymphoma; (3) strain of mice—this influence was particularly marked in dba mice, less in CFW; (4) time factor—administration of NCG before and not after RCG.

Since it has been found that RCG condensed in clumps and stored in macrophages acted on tumor cells more slowly than the same dose of the original RCG preparation (perhaps, because it has to be disintegrated and resuspended in the fluid in order to exert an effect on cells), the attenuating influence of NCG pre-treatment on RCG effect may be attributed to a shift of a large proportion of injected RCG from the fluid into numerous macrophages. This interpretation was tested in a special series of experiments which will be reported separately.

Summary and conclusions. (1) Sarcoma 37 and malignant lymphoma were cultured as free cells in the peritoneal fluid of mice by serial transfers of these cells in the peritoneal fluid. Requisite number of tumor cells (100,000 or 1,000,000) were inoculated i.p into several groups of mice (S-37 in CFW or dba strain; lymphoma in dba mice only) and treated, after 2 to 14 days, with various doses (0.05 to 0.3 mc) of radioactive colloidal gold, either intraperitoneally or intravenously. In several series of experiments the mice were pre-treated with inactive (decayed) colloidal gold (0.1 or 0.2 cc) before the treatment with active gold. Control experiments investigated the growth characteristics of S-37 and lymphoma cells in untreated mice, the effect of the treatment with active or inactive gold in normal mice and the effect on tumor cells of radioactive gold stored in macrophages. (2) The experiments have outlined the role of the following factors in production of a radiotherapeutic effect of the radioactive colloidal gold on free tumor cells growing in the peritoneal fluid; (a) strain of the tumor; (b) strain of mice; (c) size of the inoculum; (d) interval between the inoculation and the treatment; (e) route of treatment; (f) dose of radioactivity; (g) pre-treatment with inactive gold. The effect was estimated by checking on the 6th day after the treatment, the percentage of surviving mice and the presence of tumor cells in the peritoneal fluid of treated mice and of controls. (3) The results were interpreted in the light of the data on growth characteristics of tumor cells and macrophagic reaction in the peritoneal fluid.

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Radioactive Colloidal Gold in Macrophages and Serous Exudate in Peritoneal Fluid of Sarcoma Bearing Mouse.* (18530)

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Studies by Hahn, Sheppard and their associates(1) in humans and dogs, by Barrow and associates(2) in rabbits and by Sheppard and Furth(3) in mice showed that intravenously injected gold colloids are stored mainly in the macrophages (reticulo-endothelial element) of the spleen and the liver.

* This work was carried out under Contract At-(40-1)269 with the Division of Biology and Medicine, U. S. Atomic Energy Commission.

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2. Barrow, J., Tullis, J. L., and Chambers, F. F., *Nav. Med. Rev. Inst.*, Bethesda, Md., Prog. AM007039, rep. No. 24, July 25, 1949.

This intracellular storage is recognized also by microscopic examination of tissue section of these organs: the injected ultramicroscopic colloidal gold particles appear condensed in large clumps inside the liver and spleen macrophages (Barrow and associates)(2). This phenomenon of intracellular condensation of colloidal gold (either radioactive or non-active through decay) was studied recently in free macrophages which appear in large number in the peritoneal fluid of the mouse inoculated intraperitoneally with sarcoma cells and treated with radioactive or inactive gold (Goldie and Hahn)(4). In no instance were the gold clumps found in other cells of the peritoneal fluid, *i.e.*, polymorphonuclears, lymphocytes and tumor cells. Thus, the intraperitoneally injected radioactive colloidal gold is evidently distributed between serous exudate (where the introduced ultramicroscopic colloidal particles are resuspended) and macrophages (where they are condensed and stored). Recently(5) it has been found that a preparative intraperitoneal injection of inactive (decayed) colloidal gold decreased the radiotherapeutic effect of radioactive colloidal gold on free sarcoma cells in CFW mice and even more in dba mice. It appeared therefore that distribution studies of radioactive colloidal gold in the peritoneal fluid of the mouse may contribute to the understanding of the therapeutic effect of this radio-isotope on free tumor cells growing in the peritoneal fluid.

Material and methods. (A) *Radioisotope preparation.* The sol of radioactive metallic gold used was that introduced by Hahn and Sheppard(6) for treatment of malignant tumors and of leukemias.† This preparation will be referred to below as RCG. A standard dose of 0.1 mc was used throughout in most experiments: this dose destroyed all peritoneal sarcoma cells only in a minority of CFW mice, but damaged a considerable number of tumor cells in most mice(4,5). The dose of 0.3 mc was used in 2 experiments for purpose of comparison. The preparation was injected either intraperitoneally or intravenously. The RCG preparation was used when it was completely decayed by aging as inactive colloidal gold (NCG) (containing approximately 3 mg Au per ml) in doses of 0.1 or 0.2 cc injected always intraperitoneally. (B) *Biological test material.* Two different strains of mice, CFW and dba, were used for comparative study of RCG distribution: both strains were susceptible to grow free Sarcoma 37 cells in their peritoneal fluid and to produce an abundant macrophagic reaction during this growth and after radioactive treatment. The technic of culture of free tumor cells by serial transfers in the peritoneal fluid and the use of this technic for screening the effect of radioactive isotopes on tumor cells were described elsewhere(4). Standard dose of 100,000 sarcoma cells was used throughout for intraperitoneal inoculation. (C) *Technic of experiments.* In each experiment, groups of CFW and dba mice were used on the 5th or 6th day after inoculation when they had accumulated a large amount of peritoneal fluid containing numerous tumor cells and macrophages. Each experiment included 4 groups of 10 mice each: (1) CFW mice treated i.v or i.p with RCG without preparation with NCG (CFR group); (2) CFW mice prepared intraperitoneally with NCG before (i.p or i.v) injection of RCG (CFN mice); (3) dba mice treated with RCG alone (dbR group); (4) dba mice prepared by NCG and treated by RCG (dbN group). The experiments varied according to the route of RCG administration, intraperitoneal or intravenous. For checking the results, small amounts (about 0.05 cc) of peritoneal fluid were withdrawn from injected mice with capillary glass pipettes. Each specimen was immediately diluted with 5% sodium oxalate solution and centrifuged in order to separate the cellular content of the specimens from its diluted

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6. Hahn, P. F., and Sheppard, C. W., *Southern Med. J.*, 1946, v39, 558.

† Colloidal radioactive gold containing Au^{198} was obtained from Abbott Laboratories, North Chicago, Ill.

serous exudate remaining in the supernatant. The two fractions were separated, each placed in a tin cup and dried. The amount of radioactivity in each specimen was estimated by a Scaling circuit in conjunction with a thin mica end-window Geiger-Muller tube. It might be presumed that centrifugation will precipitate not only cellular elements of the peritoneal fluid but also a significant number of colloidal gold particles suspended in a protein solution. In order to test this hypothesis we have added test doses of RCG (0.3 mc) to a 10% solution of cell free exudate from the peritoneal fluid. This solution was centrifuged and the obtained fraction was tested for radioactivity. Specimens of peritoneal fluid were withdrawn from injected mice according to a standard schedule after ½ hour, 1 hour, 3, 5, 7, 10 and 24 hours. In some experiments sampling was done only after 24 and 48 hours, since frequently repeated withdrawal of the fluid from the same mouse limited its usefulness. In at least one experiment on each type of treatment (i.p or i.v) specimens of peritoneal fluid taken from treated mice were examined partly for radioactivity and partly for integrity of tumor cells and for condensation of RCG in macrophages.

The aim of the experiment was to determine the relative portion of radioactivity in each of 2 fractions of centrifuged fluid, *i.e.*, to calculate the ratio P/S (radioactivity of precipitate/radioactivity of supernatant) for each specimen, independently from the absolute radioactivity content in this specimen. For this reason, in all collected specimens from the same series of mice (same experiment), the radioactivity was tested on the same day and approximately on the same hour: absolute values of radioactivity were decreased in all specimens by progressive decay of the radioisotope, but the ratios remained unchanged.

Results. Centrifugation precipitated from RG suspension in diluted serous exudate only an insignificant amount of radioactive material which could not influence our results showing nearly always a ratio much higher than 1.0.

TABLE I. Distribution of Radioactivity Between the Precipitate and the Supernatant (Ratio P/S) in the Peritoneal Fluid of the Mouse 24 and 48 Hours After Intraperitoneal or Intravenous Injection of RCG.

Groups of mice*	Intrap. after 24 hr	Intrap. after 48 hr	Intrav. after 24 hr	Intrav. after 48 hr	Remarks
		Dose of 0.1 mc			
CFR	12.8/1	21.8/1	1.5/1	3.5/1	Each ratio represents avg for 10 mice
CFN	26/1	28/1	4.5/1	10.5/1	
dbR	24/1	33/1	3.5/1	7.5/1	
dbN	40/1	47/1	18.5/1	35.0/1	
		Dose of 0.3 mc			
CFR	52/1	71/1			for 5 mice
CFN	60/1	68/1			
dbR	88/1	130/1			
dbN	182/1	212/1			

* See technic of exp.

The ratio P/S for specimens of peritoneal fluid withdrawn 24 and 48 hours after i.p. or i.v injection of RCG in different groups of mice is shown in Table I.

The results of Table I were partly interpolated by studying for similar experimental groups of mice, the changes of P/S ratio in the peritoneal fluid at various intervals during the first 24 hours after injection of RCG. The data obtained were plotted in graphs (Fig. 1 and 2).

Microscopic examination of peritoneal fluid repeated at hourly intervals revealed that both after intraperitoneal and intravenous injection of RCG, the damage to tumor cells (swelling, clumping of chromosomes, abnormal mitoses) appeared regularly after 4 to 5 hours and increased progressively in proportion to the dose injected, showing also individual variations in various mice.

Discussion. Dilution and centrifugation of the colloidal preparation used in these experiments did not result in precipitation of any significant amount of radioactive material. Thus, the relatively high content of radioactivity in the precipitate of the peritoneal fluid should be attributed to absorption (or adsorption) of the colloid by cellular elements of the fluid. It has been found that RCG is selectively absorbed and stored by macrophages of the peritoneal fluid in amounts so large as to be visible with low power microscope. Any adsorption of ultramicroscopic colloidal particles on the surface of other fluid

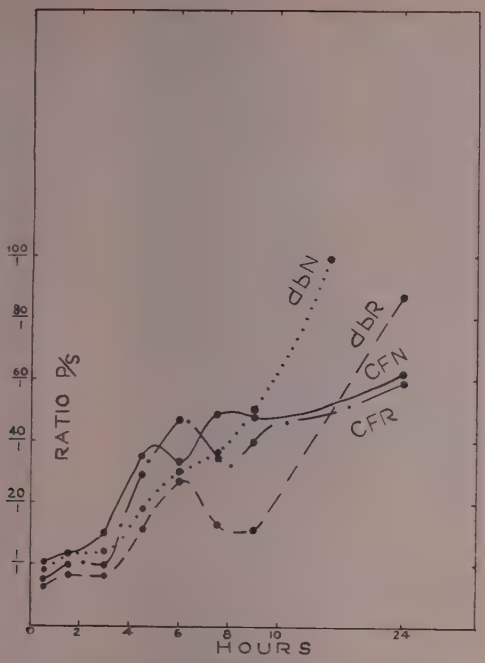

FIG. 1.

Distribution of radioactivity between precipitate and supernatant (ratio P/S) of the peritoneal fluid at various intervals after intraper. inj. of 0.1 radioactive colloidal gold into the mouse. *CFR and dbR*: groups of CFW and dba mice treated with radioactive gold only. *CFN and dbN*: groups of CFW and dba mice prepared with inactive gold before the treatment with radioactive gold.

cells appears negligible by comparison with copious storage of large gold clumps in macrophages. Thus, for all practical purposes, the P/S ratio of the peritoneal fluid (or of the blood) probably reflects the distribution of radioactive material between macrophages and serous exudate.

It should be emphasized that RCG suspended in the form of ultramicroscopic particles in the peritoneal exudate diffuses rapidly into the blood and vice versa, while RCG stored in macrophages remains localized with these cells in the peritoneal cavity. Thus, the increase of P/S in the peritoneal fluid may reflect an increased activity or/and number of macrophages absorbing the colloid from the serous exudate or a decreased diffusion of RCG from this serous exudate into the blood, or both phenomena simultaneously. On the other hand, a decrease of P/S may be attributed to a decrease in activity of macrophages or to an increase in RCG diffusion from the peritoneal fluid into the blood or to both phenomena.

It is obvious that intraperitoneally injected RCG disappears steadily and at a progressively increasing rate from the serous exudate through absorption by macrophages and simultaneously through diffusion into the blood. These two parallel phenomena account sufficiently for steady increase of P/S in all groups injected intraperitoneally (Fig. 1). On the contrary, the results of intravenous injection of RCG were determined by the balance of two opposite phenomena: RCG supply from the blood to the fluid and absorption of supplied radioactive material by macrophages. For dba mice (dbN and dbR) (Fig. 2) this balance was, during the first 4 hours, in favor of absorption by macrophages as shown by the sharp rise of P/S. This initial rise was followed by a temporary fall of P/S, reflecting presumably a transient de-

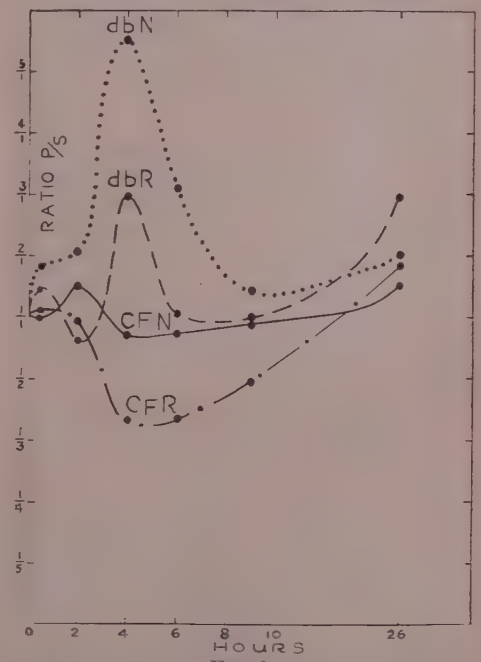

FIG. 2.

Distribution of radioactivity between precipitate and supernatant (ratio P/S) of peritoneal fluid at various intervals after intrav. inj. of 0.1 radioactive colloidal gold into the mouse. See Fig. 1.

crease in the activity of macrophages. For CFW mice (CFN and CFR) the absorption was evidently balanced by diffusion during the first 24 hours. Between 24 and 48 hours the decreased supply of RCG from the blood and the increased activity of macrophages in the peritoneal fluid raised P/S for all groups of mice to higher level (Table I).

Table I and Fig. 1 and 2 indicate clearly that higher levels of P/S were induced consistently in mice by the following factors: (1) high dose of injected RCG; (2) strain characteristics of dba mice and (3) preparation by NCG. It is easier to explain the effect of these factors by stimulation of macrophages or increase in their number than by increased diffusion of RCG into the blood. Moreover, microscopic examination showed higher percentage of macrophages in dba mice than in CFW and in mice prepared with NCG than in unprepared. Whatever may be the interpretation of effect of these factors, their role indicates the importance of various biological conditions rather than purely physical conditions in distribution of RCG in the peritoneal fluid. This interpretation is in agreement with the data of H. P. Smith(7) on the distribution of colloidal dyes between blood and tissues.

Summary and conclusions. (1) CFW and dba mice were inoculated intraperitoneally with Sarcoma 37 cells from serial intraperitoneal transfers. After formation of copious peritoneal exudate containing tumor cells and macrophages, these mice received intraperitoneally or intravenously 0.1 mc (in 2 series 0.3 mc) of radioactive colloidal gold. At various intervals (½ hour to 48 hours) after injection, specimens of peritoneal fluid were withdrawn from these mice and centrifuged. Radioactivity of precipitate (P) and of supernatant (S) in each specimen was determined by Geiger counter. (2) Ratio P/S was accepted as index of radioactivity distribution between macrophages and serous exudate of the peritoneal fluid. Data illustrating trends in changes of P/S in various groups of mice were plotted in graphs and discussed. These data were interpreted as the combined effect of two parallel phenomena responsible for disappearance of radioactive colloid from the peritoneal serous exudate; (a) absorption and storage of colloid particles into macrophages; (b) their diffusion into circulating blood; (3) The ratio P/S in various specimens of peritoneal fluid from the same mouse remained consistently on higher levels after (a) the use of higher doses of radioactive colloidal gold; (b) the use of dba mice and (c) the preparation with inactive (decayed) colloidal gold before treatment with radioactive gold. The effect of these factors is attributed to the increase in the number and in the activity of macrophages.

7. Smith, H. P., *J. Exp. Med.*, 1930, v51, 309, 395.

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Pneumoperitoneum in Preoperative Preparation for Total Gastrectomy. (18531)

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Inasmuch as pneumoperitoneum, employed in the therapy of human tuberculosis, produces a rise of the diaphragm and a visceroptosis, it seemed logical that pneumoperitoneum might be of value in making the stomach and the lower segment of the esophagus more accessible for the procedure of total gastrectomy, if it were employed prior to the exploratory laparotomy. However, it was not certain that this alteration of organ position would persist after the peritoneum had been entered. Experimental studies were thus undertaken.

Eight adult mongrel dogs were employed in this study. Four dogs were employed as

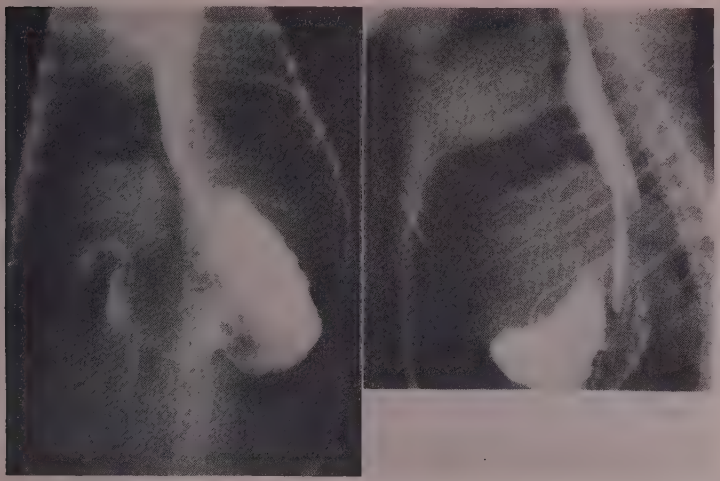

FIG. 1(A.B.).

A.P. and lateral roentgenograms of the upper abdomen and lower chest of a dog subjected to pneumoperitoneum. Barium was introduced through a catheter. The long segment of intra-abdominal esophagus is quite apparent. The stomach is well below the costal margin. (The right costal margin is easily seen). The spleen and superior surface of the liver are at the costal margin.

controls. A pneumoperitoneum was established in an experimental group of 4 dogs. A No. 18 gauge needle was passed through the abdominal wall of the left hyperchrondrium into the peritoneal cavity employing 10 cc of 1% procaine local anesthesia. 1500 cc of air were introduced with a 50 cc syringe and a 3-way stopcock. A week later 1000 cc of air were similarly introduced into the peritoneal cavity. A week later the dogs were anesthetized with intravenous injections of nembutal. AP and left lateral chest x-rays were taken of the experimental group to include in the plate the upper half of the abdomen. Fig. 1. Eight dogs were subjected to exploratory laparotomy.

Results. 1. As a result of the pneumoperitoneum the diaphragms were found to have been elevated and stretched and after release of the intra-abdominal air pressure the musculature was flabby. The excursions of the stretched diaphragms were 2-3 cm greater than in the controls. In the control group the diaphragms were taut.

2. In the experimental group the liver was found suspended from the under surface of the diaphragm by a central ligament 1.5 cm in length. This contained a 1.5 cm segment of exposed inferior vena cava and a ½ cm segment of the hepatic veins as these entered the inferior vena cava. This ligament was invested by serosa and subserosa extending from the inferior surface of the diaphragm into the capsule of the liver. In the control animals the liver was found against the diaphragm suspended by the triangular and central ligaments. Only a 2-3 mm segment of the inferior vena cava could be exposed and the hepatic veins could not be visualized.

3. Caudal to the ptosed liver was the stomach. As a result of ptosis of the viscera the abdominal segment of esophagus measured 3-4 cm in length. This intra-abdominal segment of esophagus had herniated from the mediastinum into the peritoneal cavity. In the controls the abdominal segment of the esophagus was less than ½ cm in length. The 3-4 cm abdominal segment was invested by markedly thinned out diaphragmatic muscle fibers and serosa and subserosa extending in "hernia sac" fashion from the peritoneal aspect of the diaphragm. This serosal and subserosal investment of the herniated segment of the esophagus inserted into the serosa of the stomach at the cardia.

4. The cardia of the stomach was found

at the left costal margin in the experimental animals. In the controls the cardia was found 1 cm below the diaphragm; 4-5 cm cephalad to the left costal margin.

5. In the pneumoperitoneum group the spleen was found at the costal margin. As a result of the ptosis of the viscera the root of the mesentery was stretched. In the control animals the spleen was found in contact with the inferior surface of the diaphragm.

6. Since the visceroptosis had produced a long intraabdominal esophageal segment making the entire stomach readily accessible, a total gastrectomy and a conventional loop 2 layer silk esophagojejunostomy was more readily accomplished in the group of animals prepared with pneumoperitoneum. Because the intra-abdominal segment of esophagus in the pneumoperitoneum group was invested with a serosa and subserosa, the esophagojejunostomy was stronger than could be achieved in the control group since in this latter group the esophageal muscularis was not covered with serosa.

Summary and conclusion. 1. Pneumoperitoneum produces (a) an intra-abdominal herniation of the lower end of the eosphagus (b) a ptosis of the stomach, liver, spleen and the root of the mesentery, displacing the first 3 structures from beneath the costal margin and therefore making them more accessible for surgical procedures. 2. Pneumoperitoneum employed in the dog, prior to total gastric resection, proved valuable. As a result of the intra abdominal herniation of the lower segment of the esophagus, the displacement of the cardia of the stomach to the level of the costal margin, and the mobility of the root of the mesentery, the stomach was readily resected and an esophagojejunostomy established.

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Cardiac Effects of a Hypotensive Veratrum Derivative in Dogs Premedicated with Digitalis or Quinidine.* (18532)

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This investigation of the effects of Veriloid† on the electrocardiogram (EKG) of dogs premedicated with toxic dosages of digitalis or quinidine was undertaken because of a clinical impression that cardiac side actions of veratrum preparations and of digitalis or quinidine may be additive(1,2).

Method. Four series of experiments were done on adult mongrel dogs weighing 5 to 15 kg. Series I: Veriloid,‡ 1 mcg/kg/min for 10 minutes,§ was infused by vein into unanesthetized dogs. Thirty minutes from the beginning of that infusion, atropine sulfate, 0.05 to 0.25 mg/kg, was administered intravenously.

Series II: Dogs were premedicated with toxic dosage of a digitalis preparation. Drugs used were digitoxin,‡ 0.2 or 0.3 mg/kg, and ouabain,‡ 0.05 to 0.07 mg/kg. Evidence for toxic digitalization was obtained by EKG one hour after ouabain, 24 hours after digitoxin.

* This work was supported in part by a Therapeutic Research Grant from the Council on Pharmacy and Chemistry of the Amer. Medical Assn.

† Veriloid (registered trade mark of Riker Laboratories, Los Angeles) is a biologically standardized (dogs) extract of Veratrum viride of constant hypotensive potency.

1. Fries, E. D., personal communication.

2. Meilman, E., and Krayer, O., *Circulation,* 1950, v1, 204.

‡ Grateful acknowledgement is made to Abbott Laboratories for digitoxin, to E. Fougera and Co. for Ouabain Arnaud and to Riker Laboratories for Veriloid used.

§ This dose produces average fall of 30% ± 12 in mean arterial pressure with recovery in 30-90 min. in dogs anesthetized with pentobarbital; no tachyphylaxis to a second injection exists after recovery.

TABLE I. Incidence of Cardiac Rhythms in Unanesthetized Dogs After Intravenous Medication (all control rhythms sinoauricular) (SA).

Changes in rhythm	Due to: Veriloid infusion	Digitalis compounds	Digitalization followed by Veriloid infusion	Quinidine HCl	Quinidine HCl, followed by Veriloid infusion
SA, no change	22	10	6	13	15
SA to partial block	3	4	1		1
SA to complete block		2	2		
SA to ventricular		2	2	2	
Partial block, no change			2		
Complete block, no change			1		
Complete block to VT*			2		
VT, no change			2		
Partial block to SA			1		
SA to SA with Vext S†		2	1		

* VT = Ventricular tachycardia.
† Vext S = Few ventricular extrasystoles.

Veratrum and atropine sulfate were then administered as in Series I. Series III: Quinidine HCl 15 mg/kg or 25 mg/kg was infused at a rate of 5 mg/kg/min into dogs. Series IV: Animals, premedicated with quinidine HCl as in Series III, were five minutes later infused with the veratrum preparation as in Series I. Changes in cardiac-rate, rhythm and QT/RR ratio were observed in Lead II of the EKG. The stylus remained under constant observation and records were obtained at 5 minute intervals during the course of each experiment.

Results and discussion. Part I. *Effect of a veratrum derivative upon the EKG of dogs:* All dogs utilized for this study had control SA rhythm without obvious abnormality. The only changes in cardiac rhythm (Table I) in the 25 animals tested were 3 cases of AV block. The bradycardia (Table II) produced was found to be a function of the control rate (Fig. 1). The administration of atropine sulfate corrected the bradycardia and abolished the partial AV blocks. Thus the effects of Veriloid on the electrocardiogram are explicable on a basis of an increase in vagal tone.

Part II. *Effect of a veratrum derivative on the EKG of dogs premedicated with toxic dosage of digitalis:* Emesis was observed in all dogs receiving digitalis compounds (Series II) in these toxic dosages. Cardiac arrhythmias were seen in 10 of the 20 animals, (Table I) the remainder showed bradycardia (Table II). This rate reduction was found to be independent of the original rate (Fig. 1). On a basis of direct comparison of the rhythm of individual animals, it was clear that in 13 of the 20 dogs veratrum produced additive cardiac effects after digitalis (Table I). The pacemaker was displaced downward in 6; prolongation of the P-R interval was seen in 4; partial AV block in 2 and acceleration of a ventricular rhythm of 60 to 200 in one animal. In the other 7 digitalized animals no change in the rhythm occurred in 6 and 1 improved.

Further slowing of the heart occurred on administration of veratrum to the eleven digitalized animals which maintained SA rhythm (Table II). The effect of atropine sulfate in dosage of 0.05 to 1.0 mg/kg was to abolish the bradycardia produced by digitalis and the veratrum compound in those animals with an SA pacemaker, and to shift the pacemaker upward in all others.

These results are consistent with an additive action of the veratrum preparation upon the digitalized heart. Digitalis is said to exert its bradycardic action through reflex vagal stimulation and a direct action upon the myocardium(3). Veriloid, apparently by increasing the vagal tone above the level produced by digitalis, elicits a further decrease in heart rate. Vagal depression of supraventricular irritability probably accounted for the downward shifting of the pacemaker since atropine resulted in increased rates and a shift of the pacemaker upward. There were 4 dogs in which an SA rhythm was not observed following atropine sulfate after digi-

3. Gold, H., Kwit, N. T., Otto, H., and Fox, T., *J. Pharm. and Exp. Therap.*, 1939, v67, 224.

TABLE II. Heart Rate in Beats/Min. in Unanesthetized Dogs Which Maintained SA Rhythm After Various Medications.

Series	No. dogs	Control	After digitalis	After quinidine, 15 mg/kg	After quinidine, 25 mg/kg	After Veriloid*
I	25	100 ± 24				62 ± 18
II	11	102 ± 23	82 ± 23			53 ± 15
III	8	117 ± 19		165 ± 23		
	6	87 ± 19			103 ± 14	
IV	10	113 ± 19		146 ± 38		96 ± 17
	6	104 ± 12			104 ± 13	104 ± 13

talis and veratum. These may have been demonstrating a direct myocardial effect of digitalis.

Part III. *Effect of a veratrum derivative upon the EKG of dogs premedicated with quinidine hydrochloride:* The most notable change in the EKG of animals receiving only quinidine HCl in dosages of 15 or 25 mg/kg was a rise of the Q-T/R-R ratio greater than that expected for the heart rate change produced. In all records negative T waves became less negative, and upright T waves became higher. An increased heart rate was observed following the administration of quinidine, 15 mg/kg, averaging 35 beats/minute. Generalized twitching of skeletal muscle was produced. The veratrum preparation produced slowing of the heart rate in dogs premedicated with 15 mg/kg but not in those with 25 mg/kg quinidine HCl (Table II). The combination of agents produced no effects on rhythm except that one dog, demonstrating a shifting pacemaker after quinidine, converted to SA rhythm with veratrum. The T wave changes associated with the administration of quinidine tended to revert toward the control pattern after Veriloid.

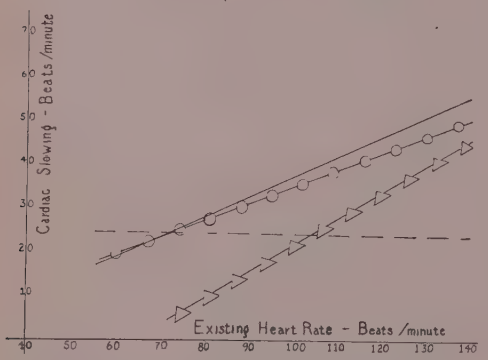

FIG. 1.

Regression lines expressing the relationship between control heart rates and heart rate decrement after intravenous medications. (For method see Snedecor, G. W., *Statistical Methods* (4th Ed.), Ames, Iowa State College Press, 1946, p. 117). ———— Veriloid, 1 mcg/kg/min. for 10 min. (SD = ±14.53, r = 0.59, t = 3.42, equation: y = —10 + 0.48x). - - - - Digitalis compound in toxic dosage (SD = ±11.3, r = 0.01, equation: y = 25 —0.008x). ○-○-○ Veriloid 1 mcg/kg/min. for 10 min. in dogs premedicated with toxic digitalis dosage. (SD = ±12.0, r = 0.61, t = 2.30, equation: y = —5 + 0.41x). Δ-Δ-Δ Veriloid 1 mcg/kg/min. for 10 min. in dogs premedicated with quinidine hydrochloride 15 mg/kg (SD = ±13.0, r = 0.89, t = 5.48, equation: y = —40 + 0.62x).

The heart is reported to be progressively blocked to vagal stimulation by quinidine(4). Since the cardiac slowing of veratrum derivatives is presumed to be a vagal effect, the absence of bradycardia from Veriloid following higher dosage of quinidine might be expected.

In this series of 66 observations with a heart rate average of 104 the normal QT/RR ratio averaged 34%. The ratio was found to vary as a straight line function of the existing rate. The same was true after the drugs with two exceptions: Dogs which received quinidine HCl alone at 25 mg/kg, and those which received veratrum and quinidine at 15 or 25 mg/kg showed an increase in the ratio greater than might have been expected from the change in heart rate.

These data do not support the current clinical concept that veratrum derivatives are contraindicated in patients receiving quinidine.

Summary. Veriloid, a reproducible hypotensive derivative of *Veratrum viride,* was

4. Hiatt, E. P., Brown, D., Quinn, G., and MacAuffie, K., *J. Pharm. and Exp. Therap.,* 1945, v85, 55.

administered in dosage of 1 mcg/kg/minute for 10 minutes to unanesthetized dogs. Similarly the veratrum derivative was administered to unanesthetized dogs premedicated with toxic dosages of digitalis preparations and to others premedicated with toxic dosages of quinidine hydrochloride. The degree of cardiac slowing from the veratrum derivative was found to be a function of pre-existing cardiac rate. No such relation was found for the bradycardia produced by digitalis. Dogs which received digitalis and veratrum showed profound bradycardia. This action appeared to be mediated by increased vagal tone since the slowing was abolished by atropine. Rhythm disturbances due to digitalis were exaggerated by veratrum. A rise in Q-T/R-R ratio above that to be expected by changing cardiac rate was associated with the administration of quinidine hydrochloride. In normal animals and in those which received digitalis, Veriloid or both, the ratio was found to vary as a straight line function of the existing heart rate. Neither quinidine alone at 15 or 25 mg/kg nor quinidine and veratrum produced appreciable incidence of arrhythmias. Cardiac slowing was observed in dogs receiving veratrum after the 15 mg/kg dose of quinidine but not after the 25 mg/kg dose.

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Action of Tri-Ethylene Melamine in Mice.* (18533)

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Cytotoxic action has been demonstrated with tri-ethylene melamine, a compound which is structurally related to the hydrolysis products of the nitrogen mustards. In a series of *in vitro* studies on lymph nodes from patients with reticulum cell sarcoma, chronic lymphatic leukemia, and lymphosarcoma, inhibition of fibroblast growth was noted(1), as well as granular disintegration of lymphocytes(2). Successful *in vivo* inhibition of certain sarcomas and leukemias in lower animals(3-7) has been obtained, as well as clinical remission in patients with malignant lymphomas(8,9) and a case of mycosis fungoides(9). Signs of toxicity were evidenced by a dramatic fall in white blood cell count(9,10), with a decrease (9,10) or an increase(9) in polymorphonuclear cells. The effect on erythrocytes was negligible, though the rat erythropoietic system was more sensitive(10). Repeated administration of effective doses at prescribed intervals was necessary in clinical application (9) and therefore the duration of toxic effect was important.

The pharmacological studies on tri-ethylene melamine were undertaken to observe the general toxic effect, as well as the latent period and duration of toxicity. The latter was of great importance since repeated administra-

* Aided, in part, by the United States Public Health Service, Research Grant C-629 (C-2), and, in part, by the American Cancer Society, DRIR-50.

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2. Plummer, J. I., Unpublished data.

3. Burchenal, J. H., Crossley, M. L., Stock, C. C., Rhodes, C. P., *Arch. Biochem.*, 1950, v26, 321.

4. Hendry, J. A., Rose, F. L., and Walpole, A. L., *V^e Congrés Internat. du Cancer,* 1950, 86-87.

5. Lewis, T. W., and Crossley M. L., *Arch. Biochem.*, 1950, v26, 319.

6. Paterson, E., and Boland, J., *V^e Congrés Internat. du Cancer,* 1950, 86.

7. Stock, C., Buckley, S. M., *V^e Congrés Internat. du Cancer,* 1950, 85.

8. Karnofsky, D. A., Burchenal, J. H., Bernstein, J. L., and Southam, C. M., *V^e Congrés Internat. du Cancer,* 1950, 185.

9. Wright, L. T., Wright, J. C., Prigot, A., and Weintraub, S., *J. Nat. Med. Assn.,* 1950, v42, 343.

10. Rose, F. L., Hendry, J. A., and Walpole, A., *Nature,* 1950, v165, 993.

TABLE I. Mortality Rate in Mature Female Albino Mice Receiving Single Injections of Tri-Ethylene Melamine Intraperitoneally.

Dose, mg/kg	Mortality	Day of death*				
		1-2	3-4	5-6	7-8	9-31
.25	1/10					1
.5	0/10					
1	0/10					
2	0/10					
3	0/11					
4	10/17		7	2	1	
5	12/17		7	4	1	
6	7/7		2	5		
7	6/6		3	3		
8	6/6		3	3		
9	6/6		3	3		
10	11/11		5	6		
20	5/5			5		
40	5/5	3	2			
80	5/5	5				
Total	74/136	8	32	31	2	1

* Day of death represents No. of days after inj.

TABLE II. Mortality Rate in Mature Female Albino Mice Receiving 2 Inj. of Tri-Ethylene Melamine Intraperitoneally.*

Each dose	Total dose, mg/kg	Mortality	Day of death†		
			5-6	7-8	9-31
.25	.5	1/4		1	
.50	1	0/5			
1	2	0/5			
2	4	0/5			
3	6	5/6		1	4
4	8	5/6			5
5	10	4/5	4		
Total		15/36	4	2	9

* Tri-ethylene melamine administered one month interval.

† Day of death represents days after second inj.

tion of sublethal doses at intervals might cause death by cumulation.

Procedure. Saline solution of tri-ethylene melamine† was administered intraperitoneally in one or two doses to female albino mice (Swiss strain). Single injections were administered at a one month interval to surviving animals, as indicated in Table II. The median lethal doses in mg/kg were calculated on the basis of size of individual dose at various intervals according to the method of Reed and Muench(11). Peripheral blood counts were taken periodically from the tail vein, using a different mouse each time.

Effect of single injection. The effect of the administration of a single dose of tri-ethylene melamine is indicated in Tables I, IV and Fig. 1. All mice receiving 80 mg/kg and three out of five of those receiving 40 mg/kg died within 24 hours. The greatest percentage of deaths occurred between the third and sixth days, with only one mortality after the eighth day (twenty-third day). The median lethal doses calculated for the second, fourth, sixth, eighth, and twenty-third days after injection (Table III) indicated a short latent period in toxic effect. Total white blood cell counts (Table IV, Fig. 1) started to decrease within twenty-four hours, especially at the higher dosage levels. A decrease in percentage of mononuclear cells occurred at that time and continued for one month after injection, even though the total white blood cell counts had returned to normal between the sixth and tenth days. This return was slower at higher doses (Table IV). The effect on the white blood cell picture was less marked at doses lower than 3 mg/kg. No effect on the erythropoietic system was observed. The mortality rate correlated with the occurrence of severe leukopenia on the third and fourth days (Fig. 1), with the greatest number of deaths occurring between the third and sixth days.

Effect of two injections. Mortalities due to the administration of the second dose at a one month interval are presented in Table II. Deaths occurred from 6 to 31 days after the second injection, with 9 out of the 15 deaths occurring after the eighth day. The median lethal doses calculated on the eighth, tenth, seventeenth, eighteenth, twenty-seventh and thirty-first days after the administration of the second dose indicated a long delay in toxic effect (Table III). Hematological changes observed were similar to those obtained with single injections, but there was no correlation between the leukopenia and mortalities.

Comparison of latency in single and double doses. Table V represents a method of comparing latency in mortality suggested by Lees

† Furnished through the courtesy of the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

11. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

TABLE III. Median Lethal Doses in mg/kg for Tri-Ethylene Melamine on Various Days After Injection.

No. of inj.	Days after inj.									
	2	4	6	8	10	17	18	23	27	31
1	40.17	4.74	4.14	4.13	4.13	4.13	4.13	4.06	4.06	4.06
2*				4.43	4.08	3.63	3.50	3.50	2.87	2.70

* Median lethal doses calculated for second inj. only.

and Lees(12). Column 7 rpresents the ratio between the death rate fifteen to thirty-one days after injection and the death rate through the eighth day. The single figure obtained in this ratio demonstrated a longer latent period after the administration of a second dose.

Discussion. Supralethal doses of tri-ethylene melamine (40 and 80 mg/kg) produced early death, whereas a latent period of from 3 to 8 days occurred at lower doses. Severe leukopenia did not occur until the third and fourth days which might have been responsible for the high percentage of mortalities at 3 to 6 days after injection, and the sharp drop in median lethal dose on the fourth day. The toxic effect of the drug seemed extinguished when the total white blood cell counts had become normal. However, the effect of the drug was not completely eliminated and its effect on the mononuclear cell count continued for one month. At this time, when a second dose was administered, it was assumed that further deaths would not be due to cumulation. The median lethal dose for the second dose was 2.5 mg/kg which was considerably lower than the 4.06 mg/kg obtained with the first dose. Even after one month, the influence of the first dose was not eradicated and thereby reduced the tolerance to the second dose. The latent period in mortalities was much longer after the second dose. Previous administration of sublethal doses may have induced a certain amount of resistance resulting in delayed mortality or there may have been a delay in the cumulative effect.

FIG. 1.

Toxic effects on mature female albino mice of single injections of 3 to 80 mg/kg of triethylene melamine.

The mortalities after single injection of a lethal dose were temporally associated with severe leukopenia whereas the mortalities after the second dose were not. This would indicate that leukopenia alone was not responsible for mortalities and the drug had a toxic effect on other tissues in addition to the hemopoietic system. This was further confirmed by the fact that a second dose was administered when the blood picture had returned to normal and animals died at a lower dosage level.

Summary and conclusions. Tri-ethylene melamine decreased the total white blood cell counts and the percentage of mononuclear cells. The total white blood cell counts returned to normal between the sixth and tenth days after injection and the mononuclear count returned to normal after one month.

12. Lees, T. W., and Lees, J. C., *Cancer,* 1950, v3, 580.

TABLE IV. Hematological Data on Mature Female Albino Mice 15 Days After Injection of Tri-Ethylene Melamine Intraperitoneally.*

Dose, mg/kg	Leukocytes†	Days after injection											
		1	2	3	4	5	6	7	9	10	11	14	15
3	WBC/mm³	5650	1600	6100	850	2350	3600	5650	5250	12800	6800		3250
	PMN %	40	27	10	33	15	15	31	51	23	37		17
	E %	6	1	0	3	0	0	0	1	2	11		1
	MNC %	54	72	90	64	85	85	69	48	75	52		82
4	WBC/mm³	4300	1650	1150	550‡			2450	5100		4500	4425	11800
	PMN %	34	57	14				35	31		65	26	25
	E %	6	0	0				0	0		0	2	1
	MNC %	60	43	86				65	69		35	72	74
5	WBC/mm³	2620	2000	650‡	350‡			1925	1200	12600		6350	15300
	PMN %	59	56					32	55	59		20	50
	E %	3	0					0	0	0		11	1
	MNC %	38	44					68	45	41		69	49
6	WBC/mm³	4100	1100		500‡								
	PMN %	50	40										
	E %	5	0										
	MNC %	45	60										
7	WBC/mm³	3750	1250	400‡									
	PMN %	37	70										
	E %	7	1										
	MNC %	56	29										
8	WBC/mm³	3050	1800										
	PMN %	37	77										
	E %	7	0										
	MNC %	56	23										
9	WBC/mm³	3200	2900										
	PMN %	83	93										
	E %	3	0										
	MNC %	14	7										
10	WBC/mm³	1600	1550	750‡									
	PMN %	67	79										
	E %	1	2										
	MNC %	32	19										
20	WBC/mm³	1750		500‡									
	PMN %	86											
	E %	0											
	MNC %	14											
40	WBC/mm³	6100		800‡									
	PMN %	81											
	E %	1											
	MNC %	18											

* Peripheral blood counts from tail vein of a different mouse each time.
† Leukocytes: WBC = Total white blood cells
PMN = Polymorphonuclear granulocytes
E = Eosinophils
MNC = Mononuclear cells—lymphocytes and monocytes.
‡ No differential count because of scarcity of leukocytes on smears.
Controls: WBC/mm³ = 3100-12700; PMN % = 9-20; E % = 0-10; MNC % = 72-90.

The highest percentage of deaths after single injections occurred between the third and sixth days when the total white blood cell counts were lowest. Surviving animals developed a resistance to a second injection, which manifested itself in a long latent period. Nine out of fifteen of the deaths in the latter group occurred between the ninth and thirty-first days, during which period the white blood cell counts were normal. The median lethal dose after a second injection was considerably lower due to the cumulative effect. The toxic effects of tri-ethylene melamine were evidenced in the dramatic fall in white blood cell

TABLE V. Mortality Rates After Injection of Tri-Ethylene Melamine.*

Drug	Each dose, mg/kg	Proportion dying, 0-8th day	Mortality rate, 0-8th day	Proportion dying, 15-31st day	Mortality rate, 15-31st day	Column 6 / Column 4
Column 1	2	3	4	5	6	7†
Tri-ethylene	.25	1/4		0/3		
melamine	3	1/6		4/5		
in 2 doses	4	0/6		2/3		
	5	4/5		0/1		
		6/21	.29	6/12	.50	+1.72
Tri-ethylene	4	10/17		0/7		
melamine	5	12/17		0/5		
in 1 dose						
		32/34	.97	0/12	0	0

* Only data satisfying the following conditions used: Dose caused 20% or more deaths; dose did not cause more than 80% deaths during first 8 days.

† Column 7 represents: Death rate for later period—15-31st (Column 6) over death rate for earlier period—0-8th days (Column 4).

count and decrease in percentage of mononuclear cells. However, the return to a normal blood picture was no indication that the effects of the drug had been extinguished.

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Aureomycin and Terramycin Treatment of *Pasteurella multocida* Infection and Neomycin's *in vitro* Effects on *P. multocida*.* (18534)

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Pasteurella multocida is primarily an animal pathogen. Recent evidence, however, indicates that it is encountered in infections of man more commonly than is generally realized(1,2). For this reason the determination of its susceptibility to various antibiotics is of interest. It was shown in a comparative study, using 8 strains, that *in vitro* aureomycin, chloromycetin, terramycin, polymyxin B and penicillin are highly effective, that streptomycin is somewhat less efficacious and bacitracin ineffective against this microorganism(3). Independently, Prier(4) reported that penicillin and streptomycin are somewhat inferior to aureomycin. Little(5) and Prier (4) found aureomycin to be effective in experimental infection of 1-day-old chicks and chick embryos. The experiments to be reported here were carried out to determine (1) the prophylactic and therapeutic efficacy of terramycin in experimental *P. multocida* infection of mice, (2) the *in vivo* effectiveness of aureomycin in an experimental animal other than previously reported, namely, the mouse and (3) the *in vitro* bacteriostatic potency of neomycin, which as yet has not been investigated.

Material and methods. Of the strains of *P. multocida* available at this laboratory and

* Aided in part by a grant of Lederle Laboratories Division, American Cyanamid Co.

1. Needham, G. M., *Proc. Staff Meetings Mayo Clinic,* 1948, v23, 361.

2. Needham, G. M., personal communication, June 7, 1950.

3. Neter, E., and Gorzynski, G. A., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 328.

4. Prier, J. E., *Vet. Med.,* 1950, v45, 243.

5. Little, P. A., *Annals N. Y. Acad. Sci.,* 1948, v51, 246.

TABLE I. Prophylactic and Therapeutic Effects of Aureomycin and Terramycin in Experimental *Pasteurella multocida* Infection of Mice.

Drug	Dosage (mg)	No. of doses (q. 48 hr)	Proportion of animals dead on days after infection			
			3rd	6th	10th	15th
Aureomycin	2	1 P*	4/64†	7/64	12/64	13/64
	2	4 P	0/72	0/72	1/72	2/72
	2	1 T‡	0/61	2/61	2/61	3/61
	2	4 T	1/62	8/62	11/62	19/62
	2	2 P	2/44	2/44	3/44	4/44
	2	2 T	7/52	13/52	16/52	17/52
	1	1 P	3/58	25/58	32/58	33/58
	1	1 T	0/39	2/39	16/39	31/39
	.5	1 P	5/34	30/34	31/34	31/34
	.5	1 T	5/34	23/34	26/34	26/34
	.2	2 P	32/60	36/60	37/60	37/60
Terramycin	2	1 P	7/108	25/108	51/108	54/108
	2	4 P	13/119	22/119	34/119	45/119
	2	1 T	0/150	35/150	54/150	82/150
	2	4 T	12/129	49/129	68/129	73/129
	1	1 P	45/122	93/122	110/122	120/122
	1	4 P	20/51	21/51	35/51	41/51
	1	1 T	15/102	73/102	75/102	75/102
	1	4 T	3/57	5/57	9/57	10/57
	.5	1 P	34/54	46/54	47/54	50/54
	.5	4 P	19/22	19/22	19/22	19/22
	.5	1 T	33/54	45/54	47/54	47/54
	.5	4 T	16/22	17/22	19/22	19/22
	.2	1 P	37/40	38/40	38/40	38/40
	.2	4 P	35/36	35/36	35/36	35/36
	.2	1 T	20/20	20/20	20/20	20/20
	.2	4 T	43/56	46/56	46/56	46/56
Control	—	—	212/212	212/212	212/212	212/212

*P = First dose of antibiotic at time of infection.
† Numerator = No. of dead animals. Denominator = Total No. of animals.
‡T = First dose of antibiotic 4 hr after infection.

tested for their sensitivity to 7 antibiotics several proved to be of low virulence in mice, while others, even in small numbers, produced a rapidly fatal infection. Strain No. 200, kindly supplied by Dr. Brunner of Cornell University and identified as *P. avicida,* was used throughout this study. It was maintained on blood agar by means of weekly transfers. For the experimental infection 18 hour-old infusion broth cultures were used in suitable dilutions; the dilutions were prepared in infusion broth and immediately injected intraperitoneally (vol. 0.5 ml) into white mice, weighing between 15 and 18 g. Aureomycin hydrochloride (intravenous) was made available through the courtesy of Dr. B. W. Carey of Lederle Laboratories, terramycin hydrochloride was kindly supplied by Dr. Elliott R. Weyer of Chas. Pfizer and Co. and neomycin sulfate by Dr. H. F. Colfer of Merck and Co. The drugs were dissolved in physiological saline solution and used within a few hours; they were administered subcutaneously (vol. 0.2 ml) in order to preclude direct contact between the antibiotic and the injected organisms. The determination of the *in vitro* bacteriostatic potency of neomycin on 8 strains of *P. multocida* was carried out according to the method previously described (3).

Results. Preliminary to the study on the *in vivo* efficacy of terramycin and aureomycin in mice infected experimentally with *P. multocida,* the MLD_{100} and MLD_{50} were determined. On the basis of experiments on 312 mice it was found that 0.5 ml of an 18-hour broth culture diluted up to 1×10^8 caused death of all animals within 3 days and that 0.5 ml of such a broth culture diluted 1 to 5×10^{10} caused death in approximately 50% of the animals. The majority of experiments with antibiotics were carried out with a broth

culture diluted 100,000 fold; the infected inoculum, therefore, contained approximately 1,000 MLD_{100} or 100,000 to 500,000 MLD_{50}. The results of aureomycin and terramycin in experimental *P. multocida* infection of white mice are summarized in Table I. A perusal of this table indicates the following: (1) All untreated animals died within 3 days; the majority succumbed between 24 and 48 hours following the infection. (2) Both antibiotics are effective in delaying death and lowering the death rate during the 15-day period of observation. (3) Aureomycin is distinctly superior to terramycin, inasmuch as only 58 (ca 16%) out of 355 mice treated with aureomycin in a dosage of 2 mg (133 mg/kg) (given once, twice and 4 times) succumbed, whereas 254 (ca 50%) out of 506 animals died when treated with terramycin. With a single 2 mg dose, the respective figures are 16 (ca 13%) out of 125 mice treated with aureomycin and 136 (ca 53%) out of 258 animals treated with terramycin.† (4) That a single 2 mg dose, prophylactically or therapeutically, is superior to single 1 mg and 0.5 mg doses with both antibiotics is evident from the fact that the fatality rates for aureomycin are respectively 13%, 66%, and 84% and those for terramycin 53%, 87%, and 90%.† These figures also reveal the greater efficacy of aureomycin in the 2 mg and 1 mg doses.† (5) Delay of treatment for 4 hours after the infection does not materially affect the outcome. (6) Treatment for 6 days (4 doses 48 hours apart) does not substantially increase the survival rate on the 15th day when the latter is compared with that of 6th and 10th day of the groups of animals which received only a single dose of the antibiotic on the day of the infection. This data together with that obtained by Little(5) and Prier(4) suggests that aureomycin may be useful in the treatment of human *P. multocida* infections, which appear to be more common than is presently realized. The therapeutic efficacy of this antibiotic in a localized *P. multocida* infection of man is being reported elsewhere(6).

Since it was observed recently in a comparative study on the bacteriostatic activity of 7 antibiotics upon 8 representative strains of *P. multocida* that streptomycin was somewhat less effective than aureomycin, chloromycetin, terramycin, polymyxin B and penicillin, it was deemed of interest to determine the growth inhibitory activity of neomycin upon these strains. Using the technic previously described(3), it was found that the bacteriostatic potency of neomycin was of the same order of magnitude as that of streptomycin, inasmuch as the minimal inhibitory concentration of neomycin ranged from 6 to 25 μg per ml and that of streptomycin was 10 μg/ml with 7 out of 8 strains studied. The eighth strain was highly resistant to streptomycin, growing profusely in the presence of 10,000-50,000 μg per ml; the patient from whom this strain was isolated had not previously received streptomycin. In contrast, this strain was susceptible to neomycin; 0.3 μg/ml prevented its growth.

Summary. (1) Both aureomycin and terramycin administered subcutaneously, either prophylactically or therapeutically (4 hours after infection) to white mice infected intraperitoneally with 1,000 MLD_{100} of *P. multocida* prolonged survival and lowered the fatility rate. Aureomycin was distinctly superior to terramycin; a single dose of 2 mg (133 mg/kg) of aureomycin resulted in a survival rate of approximately 87%, whereas that of the mice treated with terramycin was approximately 47%. Smaller doses (1 mg and less) of both antibiotics were less effective. Treatment over a period of 6 days did not increase substantially the survival rate as compared to a single dose treatment on day of infection. (2) *In vitro* neomycin was of the same bacteriostatic potency as streptomycin against 7 strains of *P. multocida* and was markedly superior against the eighth, streptomycin-resistant, strain.

† The above corresponding figures were analyzed statistically, and found to be significantly different, by John Neter of Syracuse University, to whom the authors are indebted.

6. Neter, E., DeKleine, E. H., and Egan, R. W., *J. Ped.*, 1951, v38, 242.

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Effect of Certain Antihistaminic and Steroid Compounds on Appendical Peritonitis.* (18535)

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Since Kay and Lockwood(1) suggested the relationship between serum antiproteolytic level and the mortality of experimental appendical peritonitis, we have been looking for substances which might be effective against this disease. Ungar(2) has shown that proteolysis normally precedes the formation of histamine. For this reason, it occurred to us that various agents which might conceivably act at some point during that reaction which culminates in the release of histamine should be tried in peritonitis. We chose 4 substances, one an antihistamine Diphenhydramine Hydrochloride (Benadryl†) and 3 steroids, pregnenalone,‡ allopregnane-21-OL3, 20 dione 21 acetate (allopregnane§) and cortisone acetate‖ (Cortone).

The results of our use of these 4 compounds are here reported.

Technic. Peritonitis was produced in dogs ranging from 6 to 10 kg by the same technic as previously reported by one of the authors (Kay)(3). Postoperatively the animals were started on medication immediately and were given water to drink ad lib. No I.V. fluids were given and food was withheld for 3 days. Animals which died were autopsied immediately. Surviving animals were sacrificed on the fourteenth postoperative day and autopsied.

Dosage: Benadryl was given in doses of 5 mg per kilo every eight hours or 15 mg/kilo per day. Allopregnane was given in doses of 4 mg per kilo every 6 hours or 24 mg/kilo per day. Pregnenalone was given in doses of 20 mg per kilo every 6 hours or 120 mg/kilo per day. Cortone was given in doses of 2.5 mg per kilo every 6 hours or 10 mg per kilo per day. All medications were given intramuscularly.

Results. Ten animals were studied in each of 5 groups. In the control group the mortality was 7 out of 10. In the Benadryl group, also 7 died and 3 survived. With cortone, allopregnane, and pregnenolone, the mortality was the same in each case, 8 animals died and 2 survived. Table I below shows the day of death of each animal in each group. Animals surviving 14 days are those counted as survivals and were sacrificed on that day. The group average for days survived is recorded in the final column. There is no statistically significant difference between the groups.

Discussion. Although, we realize that a certain period of time is necessary before the steroids become effective, we believe the data reported significant because medication was begun immediately postoperatively or 12 to 24 hours before the onset of peritonitis. Furthermore it was our desire to evaluate these drugs as they might be used therapeutically rather than prophylactically. The possibility remains that the steroids might be effective if given in courses before the onset of peritonitis. During the course of our work, a report of Habif *et al.*(4) appeared which described a case of perforation of gastric ulcer which occurred following a course of ACTH. The patient showed no systemic signs of peritonitis. Our work does not refute such a possibility but suggests that the 3 steroids studied have no place in the therapy of uncomplicated experimental appendical peritonitis. The observation that these agents do not affect the mortality rate in this disease

* Aided by a grant from the Warren-Teed Products Co., Columbus, Ohio.

1. Kay, J., and Lockwood, J., *Surgery,* 1947, v21, 155.

2. Ungar, G., and Mist, S., *J. Exp. Med.,* 1949, v90, 39.

† Supplied by Parke-Davis Co.

‡ Supplied by Warren-Teed Products Co.

§ Supplied by Wyeth, Inc.

‖ Supplied by Merck and Co.

3. Kay, J., and Lockwood, J., *Surgery,* 1946, v20, 56.

4. Habif, D., Hare, C., Gloser, G., *J.A.M.A.,* 1950, v144, 996.

TABLE I. Days Survival.

Treatment	Day of death														Group avg
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Control	1	5	1											3	5.6
Benadryl	2	4				1								3	5.8
Pregnenolone	1	2	3	1	1									2	5.1
Allopregnane	1	2	1	2	1			1						2	5.7
Cortisone	2	2	1		1	2								2	5.4

suggests the possibility that the observed proteolysis in peritonitis is not produced in a manner analogous to the production of fibrinolysin by specific antigens.

Conclusion. Benadryl, an antihistamine and three steroids, cortone, allopregnane and pregnenalone do not affect the course of experimental appendical peritonitis in dogs.

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Effects of Methoxinine in the Mouse.* (18536)

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Roblin *et al.*(1) showed methoxinine to be a bacteriostatic agent for *E. coli* and *Staph. aureus*, and the growth inhibition caused by it to be reversed by L-, but not by D-, methionine. Shaffer and Critchfield(2) reported the alleviation, by methionine, of the toxic effects of methoxinine in rats. These latter investigators claimed that methoxinine exerted lipotropic activity, but they failed to take into consideration the effect of the caloric restriction imposed by the methoxinine. Best(3) warns that "another pitfall . . . is that of the 'pseudolipotropic' effect of a low caloric intake." In connection with other studies being carried out in this laboratory, it was of interest to study the effects of methoxinine in mice. Some of the data seemed to clarify the "apparent" lipotropic activity of methoxinine, and are reported here.

The basal diet used in these studies had the composition shown in Table I. All additions were made at the expense of sucrose. In the first experiment, young adult male mice of the Swiss strain, in groups of 4 each, were treated as shown in Table II. Male mice of the C3H strain, 5 to 6 weeks of age, in groups of 5 each,

TABLE I. Diet Composition (per kg of diet).

	g
Soy protein*	100
L-Cystine	2.5
Salts†	50
Ruffex	20
Hydrogenated vegetable oil‡	100
Lard	50
Sucrose	677.5
	mg
Thiamine	20
Riboflavin	20
Pyridoxin	20
Calcium pantothenate	40
α-Tocopherol	40
	units
Vitamin A§	67500
Vitamin D‖	5000

* Alpha-protein, The Glidden Co., Chicago, Ill.
† Hawk-Oser salt mixture.
‡ Crisco.
§ Vit. A concentrate, Nopco Chemical Co., Harrison, N. J.
‖ Drisdol, Winthrop-Stearns, New York.

* This investigation was supported (in part) by a research grant from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service.

1. Roblin, R. O., Jr., Lampen, J. O., English, J. P., Cole, Q. P., and Vaughan, J. R., Jr., *J. Am. Chem. Soc.*, 1945, v67, 290.

2. Shaffer, C. B., and Critchfield, F. H., *J. Biol. Chem.*, 1948, v174, 489.

3. Best, C. H., *Fed. Proc.*, 1950, v9, 506.

TABLE II. Effect of Various Supplements on Weight Change and Liver Lipids of Young Adult Swiss Male Mice.

Supplement, %	Initial wt, g	Wt change, g	Total food intake, g	Liver wt, g	Liver lipids % fresh wt
—	37.5	1.6 ± 1.45*	97.2	2.95 ± 0.11*	21.0 ± 4.13*
.5 choline	37.0	1.0 ± 1.82	87.5	1.82 ± 0.26	6.4 ± 0.56
.25 methionine	36.3	—0.8 ± 1.75	87.0	2.16 ± 0.19	9.2 ± 1.34
.25 methionine + .25 methoxinine	36.3	—0.8 ± 1.36	77.1	2.24 ± 0.07	8.6 ± 1.21
.25 methoxinine	37.5	—7.8 ± 2.57	58.0	2.25 ± 0.15	7.4 ± 1.74
Basal (restricted)	37.3	—8.6 ± 3.03	59.2	1.73 ± 0.17	7.2 ± 1.10

* All values are means ± standard errors. S.E. $= \sqrt{\frac{\Sigma d^2}{n\,(n-1)}}$.

TABLE III. Effect of Various Supplements on Weight Change and Liver Lipids of Young C3H Male Mice.

Supplement, %	Initial wt, g	Wt change, g	Total food intake, g	Liver wt, g	Liver lipids % fresh wt
—	18.9	—1.3 ± 1.50*	60.6	1.78 ± 0.22*	30.8 ± 2.99*
.5 choline	18.8	2.8 ± 0.51	65.4	1.32 ± 0.04	8.2 ± 0.28
.25 methionine	18.3	2.6 ± 1.41	63.3	1.50 ± 0.16	12.9 ± 1.49
.25 methionine + .25 methoxinine	19.3	—5.0 ± 0.76	41.3	1.08 ± 0.07	7.0 ± 0.50
.25 methoxinine	19.1	—7.2 ± 0.57	28.1	0.90 ± 0.07	5.3 ± 1.45
Basal (restricted)	19.1	—7.6 ± 0.41	30.6	0.74 ± 0.18	4.7 ± 0.75
.5 methoxinine	Survival 11.6 days (4-17 days)				

* All values are means ± standard errors. S.E. $= \sqrt{\frac{\Sigma d^2}{n\,(n-1)}}$.

were similarly treated (Table III), with an additional group receiving a higher level of methoxinine. With the exception of one group in each experiment which was restricted, on the basal diet, to the food intake of the methoxinine treated animals, the food was administered *ad libitum*. Body weights and food consumption were recorded daily. After 21 days the animals were sacrificed, and the livers removed and weighed immediately. The liver lipids were determined gravimetrically, in the first experiment via chloroform extraction, and in the second experiment by extraction with a 1:1 ethanol:ether mixture.

Results. From Tables II and III it can be seen that methoxinine is toxic for mice, and that its toxic effects are alleviated by the simultaneous administration of methionine. Each of the treatments caused a lowering of liver lipids; with methionine and choline, this has been accomplished without any effect on food intake. When methoxinine was added to the diet along with methionine, there resulted a lower liver fat value, and a reduced food consumption. When the basal diet was restricted, in amount comparable to that consumed by the animals on the methoxinine supplement, there resulted a weight loss and a liver lipid value comparable to those values obtained in the methoxinine treated mice. Calculated on the basis of average body weight during the experimental period, the methoxinine intake of the mice in each of the groups receiving only the methoxinine supplement at 0.25% of the diet was of the same order, that is, 20-22 mg per 100 g of body weight per day. When methoxinine was incorporated in the diet at the 0.5% level, the survival period was short. On an intake of 45 mg per 100 g of body weight per day, one mouse survived only 4 days; on an intake of 30-32 mg per 100 g of body weight per day, the survival times of three mice were 13, 14, and 17 days.

Discussion. Neglecting the differences between the diets used, the toxicity of methoxin-

ine is of the same order in both mice and rats. Since the same general picture was obtained in both experiments with mice, it seems reasonable to assume that the effect of the methoxinine is to reduce the caloric intake, which restriction alone is capable of reducing the liver lipids to a level as low as the values obtained with choline or methionine. In the Swiss animals, an equivalent quantity of methionine offsets the effect of methoxinine. In the C3H mice, more methionine seems to be required. This is in line with our observations(4) that the C3H mice do not respond as well as Swiss mice to diets that are low in methionine, suggesting a higher requirement of the former for methionine (or protein). The basal diet, while affecting only the liver in the mouse, produced the complete choline deficiency syndrome in the weanling rat, that is, fatty liver, hemorrhagic kidneys, and intraocular hemorrhages. Littermate rats, which received methoxinine, had normal kidneys and eyes on gross examination, even though the large yellow livers indicated higher liver lipid values than were seen in the animals on the basal diet. When other littermates were fed methionine, in addition to methoxinine, the livers appeared nearer to normal.

Conclusions. 1. In mice, methoxinine causes a reduction in the food consumption and a weight loss. 2. The toxic effects of methoxinine are alleviated by methionine. 3. Methoxinine is not lipotropic in mice, since the caloric restriction imposed by its toxicity can completely account for its "apparent" lipotropic activity.

4. Travers, J. J., and Cerecedo, L. R., unpublished data.

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Effect of Locally Applied Vitamin A and Estrogen on Rat Epidermis.* (18537)

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(Introduced by Richard M. Eakin)
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Recent observations on the ability of intravaginally instilled vitamin A to partially counteract vaginal cornification induced by estrogen(1) have led us to reconsider the implication of McCullough and Dalldorf's suggestion(2) that local vitamin A deficiency is responsible for all epithelial keratinization (including that accompanying metaplasia). In particular, we were interested in investigating the possible role of local vitamin A inadequacy in normal epidermal keratinization and any interrelation between vitamin A and estrogen in this process. A relation between vitamin A and excessive epidermal keratinization has been established by observations on the syndrome of follicular hyperkeratosis accompanying vit A deficiency in man(3-5) and in the rat(6). In addition, a beneficial effect on wound healing has been reported both for vit A and for cod liver oil(7-10). However, more recently Brush

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and Lam(11) have disagreed with reports of such beneficial effects on guinea pigs using a commercial ointment containing vit A and D.

Materials and methods. Fifty-five sexually mature female rats of our Long-Evans S-1 strain were used in this study. The average age was 6½ months; variations in epidermal thickness bore no consistent relation to age differences. Littermates were divided among the several experimental groups where possible. All animals, treated and untreated, were ovariectomized 2 to 3 weeks before treatment was begun to eliminate any possible effects of cyclic estrogen production(12). They were divided into 5 groups: (a) 11 untreated control rats, (b) 11 rats treated with sesame oil alone, (c) 11 rats treated with estradiol benzoate in sesame oil, (d) 13 rats treated with pure vitamin A alcohol in sesame oil, and (e) 9 rats treated with both vitamin A and estradiol benzoate in sesame oil. Estradiol benzoate was used in a concentration of 2230 I.U./ml and the vitamin A alcohol in a concentration of 5000 I.U./ml. Extensive precautions were employed to protect the vitamin solution from the effects of light, heat and oxygen. Similarly located areas were shaved on the dorsum of each animal just posterior to the scapulae. A felt pad approximately one inch square was placed over the shaved area in all animals (including controls), and adhesive tape was bound around the rat and over the pad to keep it in place. Twice daily 0.37 ml of each oil solution was injected into the felt pad, making a total daily dose of 3700 I.U. of vitamin A to animals of groups (d) and (e), and 1650 I.U. of estradiol benzoate to those of groups (c) and (e).

After 10 days (19 applications), the animals were sacrificed, and treated segments of skin were removed and fixed for histologic study. Paraffin sections were made of Bouin's-fixed tissues for staining with hematoxylin and eosin, of chilled absolute alcohol-fixed tissues for Unna's method employing the Millon reaction for keratin(13) and for the Barger-Gomori technic for alkaline phosphatase(14). Phosphatase localization was determined on 3 untreated, 6 sesame oil treated, and 5 vitamin A treated rats using an 18-hour incubation period in glycerophosphate at pH 9.5. Control slides were run similarly in substrate poisoned with KCN. Sections were studied microscopically, and measurements of epidermal thickness were made with an ocular micrometer on the H. and E.-stained slides at 430x magnification. The thickness for each skin segment is the mean of 5 measurements made on uniform areas between hair follicles. Inasmuch as the stratum corneum was often torn away from the underlying layers and otherwise distorted, the epidermal thickness is actually that of the combined stratum germinativum (spinosum)-granulosum. In addition, granulosum thickness was measured separately.

Observations. The results of the several treatments are indicated in Fig. 1. Statistical analysis

$$\left(\text{S.E.}_m = \sqrt{\frac{\frac{\Sigma x^2}{n} - \left(\frac{\Sigma x}{n}\right)^2}{n-1}} \right)$$

shows that the 5 groups fall into two classes in so far as epidermal thickness and thickness of the stratum granulosum are concerned. At the 1% level of confidence, both the epidermis and the stratum granulosum alone in the vitamin A and estrogen-vitamin A treated animals are significantly thicker than those in the other groups.

This increased thickness is due to an increased number of cell layers in both the stratum germinativum and the granulosum,

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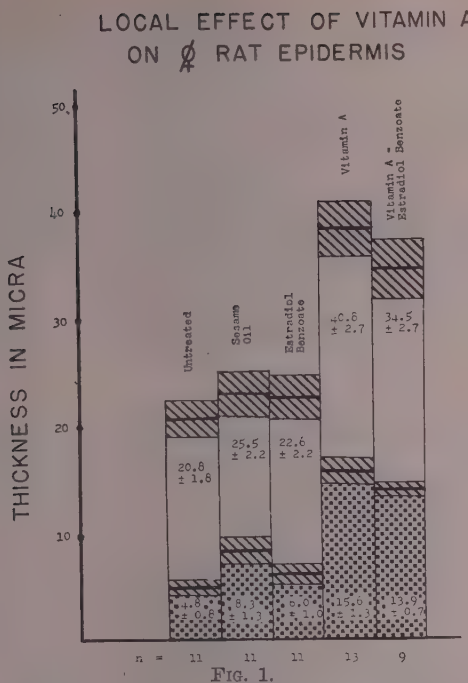

FIG. 1.

Effects of local applications of oil solutions to skin of ovariectomized female rats for 10-day period. Total height of block = mean thickness of epidermis exclusive of stratum corneum; height of stippled area = mean thickness of stratum granulosum; hatched area = standard error of mean.

as well as to an apparent increase in cell size (Fig. 5-8). Keratohyalin granules are dispersed in the cytoplasm in 3 to 5 superficial cell layers, instead of in 1 to 3 layers as occurs in those animals not treated with the vitamin (Fig. 2-4). In some vit A treated rats granules extend into an outermost layer containing spindle-shaped nuclei (Fig. 7). In these cases, all the granule-containing cells were considered as stratum granulosum, although Millon's reagent revealed a positive reaction for keratin in the most superficial layers.

The effect of vit A seemed to be entirely local; sections of skin removed from an untreated posterior region in 5 of the vit. A treated animals showed epidermal and granulosum thicknesses not significantly different from those of the untreated controls. The vitamin-treated segments in these rats showed an epidermis (exclusive of stratum corneum) 180% thicker and a stratum granulosum 420% thicker, on an average, than those of the posterior untreated segments.

The only observable effect of sesame oil and estrogen in sesame oil on the skin was an increased density of the stratum granulosum, *i.e.*, an increase in the number of discrete keratohyalin granules.

Discussion. After this study was initiated, we came across the paper of Studer and Frey (15), who reported on the effects of massive oral doses of vitamin A on immature male and female rat epidermis. They described a hypertrophy of the epidermis noticeable after three days and reaching a peak at about 9 days, with a reversion to the normal picture beginning about the 12th day and attained by about the 21st. They also stated that a similar phenomenon is observable after topical application of vit A concentrate, but supporting data were not presented in this paper. It seems to us that their description of the reversal is open to some question on the basis of the small number of animals and the lack of statistical analysis. The mean thickness reported by Studer and Frey after 11 days of treatment was 16 μ, after 22 days 13 μ, whereas the normal thickness in their immature rats was 6-10 μ. However, our observations on the local effect of vitamin A are in agreement with their description of maximal effects from orally administered vitamin. It is of real interest that Studer and Frey were able to produce a local hypervitaminosis-A in view of the presumed ability of the adult liver to store most of the orally administered vitamin(16). Both Studer and Frey's and the current observations suggest, but do not establish, the ability of vit A to counteract epidermal keratinization. This is indicated by an increase in thickness of the stratum granulosum, which may be interpreted as due to a stimulating effect of vitamin A on keratohyalin formation or to a deceleration of keratin formation. The granular gross appearance of the epidermis in most vit A treated rats may signify a sloughing of old

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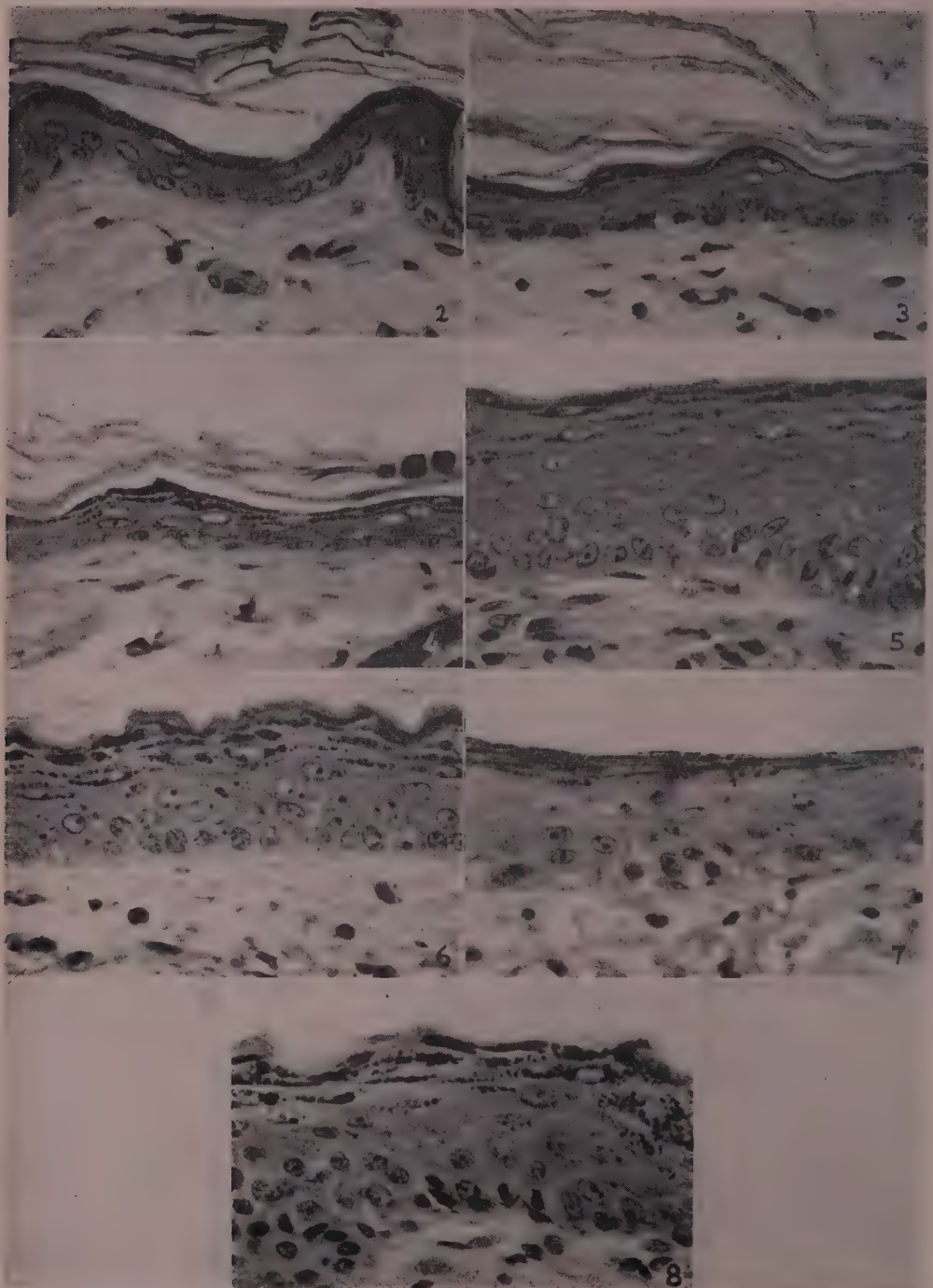

FIG. 2-8.

Photomicrographs of sections through ovariectomized female rat epidermis and underlying dermis. Bouin's fixation; H. and E.; ×480. 2. Untreated control. 3. Sesame oil treated.

4. Estradiol benzoate in sesame oil treated. 5. Vitamin A alcohol in sesame oil treated. Maximal reaction; note extensive granulosum, virtual absence of corneum. 6. Vitamin A alcohol in sesame oil treated. Note extensive granulosum, presence of corneum. 7. Vitamin A alcohol in sesame oil treated. Atypical reaction. 8. Vitamin A alcohol and estradiol benzoate in sesame oil treated. Typical vitamin A reaction.

keratin which becomes disjoined *en masse* from the underlying layers. At any rate, it can be stated with certainty that locally applied vitamin A affects epidermal growth and differentiation.

It is interesting that the epidermis, which is sensitive to deficiencies in vitamin A(3-6), has been shown to be free from the vitamin (17,18). Speculation might be allowed on the possible role of epidermal avitaminosis-A in the evolution of the amniote stratum corneum, implying that the epidermis may be unable to utilize vit A or unable to acquire sufficient quantities due to the absence of an intraepidermal vascular supply. It is also possible that the vitamin is quickly oxidized or light-inactivated in the superficial layers. Further investigations of the type described herein might aid in the validation or invalidation of such speculation.

The distribution of alkaline phosphatase differed but little in the untreated, sesame oil treated, and vit A treated skin patches. Activity was found in some dermal capillaries, particularly those immediately below the epidermis. In addition, stroma associated with sebaceous glands and hair follicles showed some activity. The activity evidenced by some sebaceous gland epithelium may have been due to diffusion. Coloration of sloughing keratin and of the stratum granulosum layer was also occasionally observed, especially in the vit A treated group. Similar coloration of the stratum granulosum has been described previously in man(19,20) and the guinea pig (21), but not in the rat(22). Inasmuch as microincineration studies in the guinea pig (21) indicated calcium deposits associated with keratohyalin, it is suggested that coloration in this area may be artifactual, due to the combination of preformed calcium deposits with phosphate produced by the action of phosphatase which leaches out into the substrate. No particular significance is attached to our observations on alkaline phosphatase activity.

Results from the topical application of estradiol benzoate indicated no appreciable influence on the epidermis (Fig. 4). The initial hypertrophy induced by estrogen in the mouse(12) was not seen after 10 days' treatment, nor was the epidermal proliferation described in postmenopausal women(23) and in senile individuals(24) supported by our observations. Similarly the degenerative effects occurring in the rat after longer treatment with estrogen(25) were not noted. Estrogen evidently had no significant effect on the proliferative changes induced by the vitamin (Fig. 8).

Summary and conclusions. Vit A administered topically to the skin for 10 days resulted in an average increase in epidermal thickness to about twice normal. A notable increase in the extent of the stratum granulosum occurred, and a decreased rate of keratin formation and/or an increased rate of keratohyalin formation are suggested. The effect was apparently entirely local; untreated skin areas from vitamin treated animals showed no significant alterations. Estrogen resulted in no observable change in the epidermis and did not counteract the stimulatory effect of the vitamin.

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Effect of Auxin on Ascorbic Oxidase Activity in Tobacco Pith Cells.* (18538)

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Much experimental work has been performed and many theories proposed in attempts to explain the mechanism of action of the plant growth hormone, 3-indoleacetic acid, or auxin, in causing cell enlargement and other growth phenomena. The subject has been carefully reviewed by Audus(1), and special aspects of the problem have been discussed by several contributors to a recent volume of review articles(2). It has been suggested that auxin causes cell enlargement by increasing the elastic or plastic extensibility of the primary wall, promoting active intussusception of new cellulose micelles into the wall, controlling cell permeability by regulating boundary potential, stimulating or reorienting respiration, causing active (*i.e.*, non-osmotic) water and solute uptake, decreasing protoplasmic viscosity, and by a number of other actions. The opinion is now widely held that the growth effects of auxin are probably due to its effect on a specific phase of cellular metabolism, quite possibly on a respiratory process. In the investigation reported here it has been found that auxin causes a striking increase in the ascorbic oxidase activity of cultured parenchyma cells of tobacco stem preceding and during their enlargement. Evidence is also presented that this enzyme is structurally associated with the cell wall. The culture of sections of pith from tobacco stems was initiated by Skoog and Tsui(3), who observed that the cells of this tissue did not enlarge appreciably on White's medium, but enlarged greatly on addition of indoleacetic acid (IAA) to the medium. Jablonski and Skoog(4) have since studied the responses of these cells to concentrations of IAA up to 20 mg/1, and have found that enlargement is slight at 0.02 mg, marked at 2.0 mg, and inhibited at 20.0 mg/1. Enlargement occurs earlier at 2.0 mg/1 than at the lower concentrations tested. Careful anatomical examination has shown that no cell divisions occur in such cultures.

Since the pith consists of a single cell type in which the hormone at physiological concentrations elicits pronounced cell enlargement uncomplicated by cell division, it appeared to be a particularly favorable material for the investigation of the specific effects of auxin on the metabolic processes involved in cell enlargement.

Materials and methods. Sections of pith approximately 1.5 x 4 x 10 mm, cut from young internodes of tobacco (Wisconsin No. 38) grown in the greenhouse, were weighed in sterile vials and cultured on a modified White's nutrient medium as described for tobacco stem callus(5). The sections were grown in 125 ml Erlenmeyer flasks with 50 ml of medium and 4 sections per flask. One-half of the cultures received indoleacetic acid at a concentration of 3.5 mg/1 (2 x 10^{-5}M), the remainder without auxin serving as controls. Six flasks with and 6 without auxin were used in each assay: 2 from each group for dry weight determinations, 2 for respiration, and 2 for the enzyme assays. Fresh weights were determined on a Roller-Smith

* This work was supported in part by a grant from the Wisconsin Alumni Research Foundation administered by the University Research Committee. Appreciation is expressed to Professor Folke Skoog for making available to the writer the wide variety of tissue cultures which have been developed in his program of growth studies. Access to these cultures led to the selection of the pith tissue for the present investigation. It is a pleasure to thank Mr. John R. Jablonski for preparing the cultures used. Thanks are also expressed to Professors Folke Skoog and Paul J. Allen for comments on the manuscript, and to the latter for performing 2 of the assays.

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TABLE I. Effects of 3.5 mg/1 IAA on Respiration and Ascorbic Oxidase Activity of Tobacco Pith Sections Grown *in Vitro*. Experiment 2.

Age of culture (days)	Respiration (μl O_2 uptake/g fresh wt/hr)		Ascorbic oxidase activity (μl O_2 uptake/g fresh wt/hr)		Q_{O_2} (ascorbate)	
	Control	IAA	Control	IAA	Control	IAA
0	31.4		369		6.5	
2	33.2	26.2	504	638	11.8	17.2
3	35.4	41.8	473	703	13.1	18.1
4	33.2	47.5	453	998	12.8	20.4
5	30.3	44.1	476	1703	12.6	45.1
6	34.8	42.6	468	2054	10.2	59.5
8	36.9	53.7	442	1860	7.9	54.3
10	42.5	55.2	428	1980	9.8	59.3
13	34.5	42.4	955	2394	19.5	78.2
16	45.1	34.6	1065	2284	21.2	73.4

torsion balance after the sections were carefully surface-dried with filter paper. Since the sections were not surface-dried for the first weighing and the surface liquid for 8 sections amounted to 80-100 mg, the initial weights set upper limits only. Oxygen consumption was determined using conventional Warburg equipment and technics. The intact sections were suspended in 2.0 ml of M/20 phosphate buffer at pH 6.0 containing 0.02 M sucrose, and their oxygen uptake was followed for 1 hour at 25°C.

For the oxidase assays, the sections were suspended in 4.0 ml of cold M/20 phosphate buffer at pH 6.0 and homogenized for 1.5 min. in a Potter-Elvehjem glass homogenizer chilled in ice water. Centrifugations were performed at 2°C in an International refrigerated centrifuge. For ascorbic oxidase assays, 1.0 ml aliquots of homogenate and 0.5 ml of 0.12 M ascorbate adjusted to pH 6.0 with NaOH were added to the central compartments of the vessels and the oxygen uptake at 25°C recorded for duplicate vessels at 10-minute intervals. Since there is no CO_2 production by the system, KOH was omitted from the insets. The ascorbate and the substrates in assays for other enzymes were prepared in M/20 phosphate of appropriate pH. It was ascertained that there is no endogenous oxygen consumption by the homogenates, and no measurable autoxidation of ascorbate in the absence of homogenate at the pH employed. Boiling the homogenate for 2 minutes destroys virtually all of the activity toward ascorbate. As has been reported previously(6), the rate of reaction is found to be independent of the ascorbate concentration between wide limits and directly proportional to the enzyme concentration.

Assays for tyrosinase employed 1.0 ml of the above homogenate and 0.5 ml of 0.06 M catechol neutralized to pH 6.0. In assaying for cytochrome oxidase, 1.0 ml of homogenate prepared in M/20 phosphate buffer at pH 7.0, 0.5 ml of 0.06 M p-phenylenediamine neutralized to pH 7.0, and 0.5 ml of 1×10^{-4} cytochrome c were used. Correction was made for oxidation of p-phenylenediamine in the absence of cytochrome c.

Results. The pith cells respond to auxin beginning within 2-3 days by an abrupt rise in ascorbic oxidase activity accompanied by a lesser respiratory increase, and subsequently by gains in fresh and dry weight.

The ascorbic oxidase activity responds strikingly to an exogenous supply of auxin (Table I, Fig. 1). The increase is evident by the 2nd day and quite rapid until the 6th, after which it appears to be more gradual. Thus in Exp. 1, the activity rose 1000% on a fresh weight basis and 1900% on a dry weight basis in the first 6 days on auxin, while it rose only 29% and 37%, respectively, in the controls. Assays beyond the 16th day indicate a gradual decline in the activity. The rise in activity of ascorbic oxidase begins be-

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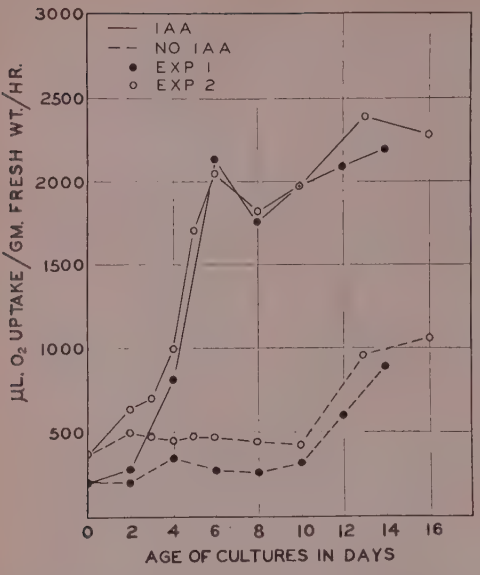

FIG. 1.

Effect of 3.5 mg/l indoleacetic acid on ascorbic oxidase activity of tobacco pith sections cultured *in vitro*. Curves based on oxygen consumption by homogenates on ascorbate.

fore any increase in respiration or weight can be detected, is most rapid either prior to or concomitant with the most rapid respiratory and weight increases, and reaches a peak after the respiratory rate has declined (Table I). The controls, on the other hand, show a constant, relatively low activity until after the 10th day, when there is a sharp rise which by the 16th day reaches a level shown by the auxin-grown tissue on the 4th day. Subsequent to the 16th day, however, the control tissue turns brown and decreases in oxidase and respiratory activity and in fresh and dry weight. The rise in the controls is being investigated further.

The respiratory rate of the pith sections on auxin shows an initial drop (Table I, 2nd day), followed by an increase until the 10th day, when a peak 76% above the initial value is reached. Thus respiration increases during the period of greatest increase in oxidase activity and gain in fresh weight. Unlike the latter two properties, it declines beyond the 10th day. The control tissue shows a lower respiratory rate which gradually increases to 46% above the initial value within 16 days.

The sections grown on auxin are not visibly different from the controls until the 4th day of culture, when they are more translucent and are bowed, with the concave face toward the agar. By the 5th day they are obviously enlarged and by the 7th or 8th, are fissured on the upper surface where groups of cells have separated. After about 2 weeks many of the individual cells become separated from their neighbors. The cells increase in size rapidly until the 10th or 12th day, after which there is little further enlargement.

The fresh and dry weight data for one experiment presented in Table II indicate the extent of growth which occurs. While there is a small amount of growth in the controls, that in the sections on auxin occurs earlier and is much greater. Jablonski and Skoog (4), using an improved method of cutting the pith sections from the plants, have reduced the variability due to tissue injury and have obtained even greater differences between control sections and those supplied with auxin.

Fractionation of the pith homogenates by differential centrifugation followed by assay and microscopic examination of the fractions indicates that the ascorbic oxidase remains attached to the wall fragments. Although the large, thin-walled cells are completely broken by a short period of homogenization, the sheets of wall material are not greatly dispersed in the Potter homogenizer. If the homogenate is then centrifuged at low speed, most of the activity is found in this sedimented wall material. For example, pith grown on IAA for 4 days was homogenized for 2 min., and the unfractionated homogenate was found to consume 1,021 μl O_2/g fresh wt/hr. The sediment equivalent to 1 g of whole homogenate, obtained by centrifuging at 500 x g for 5 min, consumed 901 μl O_2, and the equivalent supernatant, 114 μl. The recovery in the 2 fractions was 99.4% of the activity of whole homogenate, while the sediment showed 89% of the combined activity of the 2 fractions. The thermolability of the sedimented activity was shown by boiling the sediment for 2 min., after which the uptake

TABLE II. Effects of 3.5 mg/l IAA on Weights of Tobacco Pith Sections Grown *in Vitro*. Figures are values for 8 sections from 2 flasks. Experiment 2.

Age of cultures (days)	Fresh wt (mg)				Dry wt (mg)		Change in dry wt (mg)†		% dry wt	
	Control		IAA		Control	IAA	Control	IAA	Control	IAA
0		714			27.2				3.81	
2	(679)*	621	(658)*	584	26.6	21.7	— 0.6	— 5.5	4.28	3.72
3	(722)	606	(719)	663	21.8	25.7	— 5.4	— 1.5	3.60	3.88
4	(756)	641	(590)	606	22.6	29.7	— 4.6	+ 2.5	3.53	4.90
5	(614)	540	(716)	827	20.4	31.3	— 6.8	+ 4.1	3.78	3.78
6	(686)	668	(737)	785	30.7	27.1	+ 3.5	— 0.1	4.60	3.45
8	(690)	725	(678)	1223	41.7	42.0	+14.5	+14.8	5.60	3.43
10	(764)	657	(741)	953	28.6	35.2	+ 1.4	+ 8.0	4.36	3.34
13	(689)	665	(685)	1298	32.7	39.7	+ 5.5	+12.5	4.91	3.06
16	(762)	769	(753)	1248	38.5	38.8	+11.3	+11.6	5.01	3.11

* Figures in parentheses are the weights of the same 8 sections at time of planting, determined without surface-drying the sections.

† Change from average value for 8 sections of 27.2 mg based on weights of 32 sections at beginning of experiment.

was only 39 μl for the equivalent of 1 g of whole homogenate.

Even prolonged homogenization, *e.g.*, for as long as 12 min., displaces very little of the activity from the sediment into the supernatant. When the Waring Blendor is employed, however, considerably more of the activity is found in the supernatant, due presumably to a more drastic action in dispersing the cell wall and adhering cytoplasm.

The oxidase activity does not reside in plastids or nuclei. Chloroplasts are absent, and starch grains and other plastids are few. Most of these can be broken by homogenization with displacement of the fragments into the supernatant, but no appreciable fraction of the oxidase activity is transferred into the supernatant. The nuclei are large, and it has been determined that they are broken during the homogenization and that very little Feulgen-positive material is found in the sediment.† Nor can the activity be ascribed to agglutinated mitochondria which might sediment with wall material, since mitochondria centrifuged from the supernatant are without activity. Homogenates prepared in distilled water and in 0.25 M sucrose solution show a localization of activity similar to that found for homogenates prepared in phosphate buffer. The localization of the activity suggests that the enzyme may be in cytoplasm intimately associated with and perhaps interpenetrating the cell wall.

Intact cells oxidize ascorbate at a rate virtually equal to that of a homogenate of such cells. This was demonstrated with cells which had become completely separated from one another during enlargement. For example, cells 46 days old, respiring at a rate of 23 μl/g fresh wt/hr, consumed 809 μl O_2/g fresh wt/hr on ascorbate, while a homogenate of similar cells consumed 805 μl. Whether this rather surprising activity of the intact cells indicates rapid penetration of the ascorbate or localization of the enzyme at the cytoplasmic surface, where it is readily reached, has not yet been ascertained. Since ascorbate at pH 6.0 is largely dissociated ($pK_1 = 4.17$), its rapid penetration into the cell seems unlikely.

The oxygen consumed by intact cells supplied with a measured amount of ascorbate does not exceed that expected for oxidation of all the ascorbate to dehydroascorbate. In one experiment, 555 mg of cells with an initial oxygen consumption of 18 μl/hr were employed. On addition of 0.5 ml of 0.03 M ascorbate, they consumed 170 μl O_2 in 35 min., by which time the oxygen uptake had dropped to the rate before the addition of ascorbate. Corrected for respiration, the uptake on ascorbate was 160 μl, or 95% of the 168 μl expected for complete oxidation to

† The author expresses his appreciation to Mr. P. K. Nelson for examining the sediment for nuclear material.

dehydroascorbate. This indicates that there is no appreciable concomitant reduction of the dehydroascorbate, since this would be followed by re-oxidation of the ascorbate and a greater oxygen uptake than calculated. Nevertheless, in homogenates of this material, dehydroascorbate is rapidly reduced upon addition of the sulphydryl form of glutathione. The question whether an ascorbic reductase is responsible for this reaction and whether this system is similar to the one studied by Hopkins and co-workers(7-8) is being investigated.

Since auxin in high concentration inhibits plant growth, including that of the pith tissue (4), the effect of a high concentration (17.5 mg/1) in the medium on the respiration and ascorbic oxidase activity of pith cells was determined. No growth was apparent by the 5th day, and the fresh wt (449 mg for 8 sections) was below the original fresh wt (492 mg). While the respiratory rate was not noticeably decreased (O_2 uptake of 29.4 μl/g fresh wt/hr compared with 30.3 μl for the control), the ascorbic oxidase activity was strongly decreased, being only 81 μl/g fresh wt/hr compared with 476 μl for the control. Thus if the stimulation of this enzyme by auxin is important for cell growth, then its inhibition by auxin in high concentration may be the basis for the inhibition of growth by auxin.

It is an interesting fact that auxin does not stimulate the activity of ascorbic oxidase when added directly to homogenates. This was determined both on pith taken directly from tobacco plants and on that which had grown on the control medium for several days. Failure to obtain *in vitro* stimulation of this oxidase with auxin has been reported previously by Wagenknecht(9), who employed homogenates of leaves and roots of yellow wax beans.

Assays for cytochrome oxidase and tyrosinase in the pith indicate no marked response of these enzymes to auxin. Low levels of cytochrome oxidase were found in both the controls and in pith grown on auxin. Tyrosinase activity could not be demonstrated in young control or treated cultures, but after about 2 weeks it was found in both, and in increasing amounts as the tissues aged.

Discussion. While ascorbic oxidase has been reported from a number of higher plants (10,11), it may be of much more general occurrence than is apparent from the literature. The writer has observed that vigorous activity toward ascorbate is characteristic of homogenates of actively growing material, including young fern fronds, young leaves of tobacco, bean, wheat, corn, grape and onion, root tips of corn and flax seedlings, and callus tissue cultures of several species. On the other hand, the slow-growing branches of *Psilotum* have low activity, and the storage leaves of onion, none. The high ascorbic oxidase activity characteristic of young grape leaves and galls decreases markedly with age(12). Furthermore, young galls, in which the cells are enlarging greatly, have much higher activity than the contiguous leaf tissue. It appears from these facts that high ascorbic oxidase activity, like high auxin content, is associated with rapid growth. It is of particular interest in this connection that tobacco, sunflower, marigold and carrot callus tissues, which synthesize sufficient auxin to maintain a continuous high rate of growth on media without added auxin, are found to have vigorous ascorbic oxidase activity.

In all homogenates which have been fractionated by centrifugation, including those of wheat and grape leaves, corn root tips and the above-named callus tissues, the ascorbic oxidase is largely recovered in the wall fraction. While many studies of this enzyme in the past have been carried out on centrifugated saps, the presence of ascorbic oxidase in such preparations may not be inconsistent with the

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8. Crook, E. M., and Morgan, E. J., *Biochem. J.*, 1944, v38, 10.

9. Wagenknecht, A. C., abstr. program 22nd ann. mtg., Am. Soc. Plant Physiol., 1947.

10. Rothchild, M. L., and MacVicar, R., *Fed. Proc.*, 1949, v8, 245.

11. Burris, R. H., and Little, H. N., p. 166 in Respiratory Enzymes, Burgess Publ. Co., Minneapolis, 1949.

12. Newcomb, E. H., in Plant Growth Substances, University of Wisconsin Press, Madison, 1951.

present results since more drastic preparative technics than those employed here may displace considerable activity into the supernatant, even though in the intact tissues the enzyme occurs in the peripheral cytoplasm.

Ascorbic oxidase may be, of course, only one of a number of enzymes which change in amount or activity in response to auxin. The following points, however, are pertinent in this regard: (1) the magnitude of the response of ascorbic oxidase to auxin; (2) the increase in activity of this enzyme prior to a detectable increase in tissue size or weight; (3) the rapid increase in enzyme activity which in the early stages parallels the rapid cell enlargement. These facts together with demonstration of an association of the enzyme with cell wall material in several tissues and of its high activity in growing tissues of a variety of plants suggest that ascorbic oxidase is causally concerned in cell enlargement and that its synthesis or activation may be controlled by auxin generally.

The function of ascorbic oxidase in the metabolism of the plant cell is unknown, although it has frequently been suggested that this enzyme may serve as a respiratory terminal oxidase(11,13). If ascorbic oxidase functions in the pith tissue as a respiratory terminal oxidase, then its increase may represent part of a change in respiration essential to the enlargement process. It is difficult to see how a modest increase in respiration occurring in the cytoplasmic interior, *e.g.*, in mitochondria, could lead to the burst in wall growth which occurs in these parenchyma cells on exposure to auxin. Such an effect would be more understandable if the increase in respiration were localized in the cytoplasm adjacent to or interpenetrating the primary wall, since this respiration might be the immediate source of energy for wall growth, active absorption of water, or other mechanism underlying cell enlargement. For instance, the ascorbic oxidase system might serve to generate energy-rich phosphate: linkage of inorganic phosphate to ascorbate followed by oxidation of the phosphoascorbate could generate an energy-rich phosphate bond, as suggested by Lipmann(14) for compounds of the ascorbic acid type.

Another possibility is that the increase in ascorbic oxidase activity alters the properties of the plasma membrane by increasing the potential difference across the membrane. This could be accomplished by maintenance of ascorbate in the oxidized form at the cell surface if it is largely in the reduced form in the cell interior.

It is also conceivable that this enzyme plays some specific, though as yet unknown role in the organized, active growth of the primary wall which may control cell enlargement. Whatever the mechanism may be, insofar as auxin and ascorbic oxidase are causally related in growth, study of plant growth substances in terms of the action of this enzyme system constitutes a new experimental approach which may aid in elucidating the growth process.

Summary. Auxin (3-indoleacetic acid) at a concentration of 3.5 mg/l in the medium causes a striking increase in the ascorbic oxidase activity of cultured tobacco pith sections. This is subsequently accompanied by a lesser respiratory increase, by cell enlargement, and by gains in fresh and dry weight. No cell divisions occur. Since the ascorbic oxidase activity of homogenates is localized in the cell wall fraction obtained by differential centrifugation, it is suggested that the enzyme occurs in cytoplasm intimately associated with the wall. Vigorous ascorbic oxidase activity is characteristic of a variety of actively growing plant tissues examined. It is suggested that ascorbic oxidase may be causally related to cell growth.

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Received February 5, 1951. P.S.E.B.M., 1951, v76.

Effect of Desoxycorticosterone upon the Toxic Actions of Somatotrophic Hormone. (18539)

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Earlier animal experiments had shown that lyophilized anterior pituitary (LAP) material causes nephrosclerosis, polyuria as well as lesions in the cardiovascular and connective tissues which resemble those seen in the so-called "collagen diseases" of man(1,2). In these and many other respects, LAP imitated the actions of mineralo-corticoids, such as desoxycorticosterone acetate (DCA)(8) or desoxocortisone(3). Since LAP is very active in causing adrenocortical enlargement, it would have been tempting to ascribe its above mentioned "DCA-like" effects to its corticotrophin content. However, later, when adequate amounts of pure ACTH became available, it was noted that even the highest tolerable doses of the latter failed to reproduce these actions of LAP. Therefore we were forced to conclude that the mineralo-corticoid-like properties of LAP must be due to some other factor present in the pituitary preparation, which was merely referred to as the "LAP-factor" or "X-factor" of the anterior lobe(4). Since LAP is especially rich in the so-called "growth hormone" or somatotrophic hormone (STH), we compared the actions of our LAP with those of electrophoretically pure STH (kindly supplied by Professor C. H. Li of Berkeley, California). A large series of pertinent experiments showed that the latter reproduces, with great fidelity, all the DCA-like actions of the crude LAP preparations(5-7). The question arose whether STH elicits these effects by inducing the adrenal cortex to produce mineralo-corticoids—that is through a special type of corticotrophic action —or whether there is a synergism between independently produced mineralo-corticoids and STH, in the peripheral receptor cells themselves.

In the present communication we wish to report upon experiments in which STH and DCA were administered, both singly and in combination, in order to examine a possible synergism between them.

Materials and methods. Twenty-five female piebald rats weighing 85-110 g (average 97 g) were used as *experimental animals.* In all of them the right kidney was removed on the day preceding the administration of the first injections. The animals received a powdered "Purina Fox Chow" diet and were given 1% NaCl solution instead of drinking water. The unilateral nephrectomy and the NaCl supplement were indicated by earlier experiments which showed that these factors increase sensitivity to the nephrosclerotic action of LAP(4). On the day following the unilateral nephrectomy the rats were subdivided into four groups as follows: I normal controls, II received STH alone, III were given DCA alone and IV STH and DCA simultaneously but injected at different sites.

The *STH preparation* used was one supplied by N. V. Organon (Oss) bearing the lot number CH I Kr. and was prepared by Professor Dingemanse of Amsterdam (Holland). It had been sent to Dr. Pedersen of Upsala (Sweden) who examined it by the ultracentrifuge method and found it to consist of two fractions, one representing 67% and other 33% of the material. This, as well as other tests performed by Organon (Oss), led the firm to conclude that the preparation consists of 67% STH and 33% of contaminating material. This preparation was dissolved in 1% NaCl, in such a manner that each individual dose was contained in 0.2 cc of the solvent. 5 mg per day, divided into 3 subcutaneous injections, were given once every 8 hours

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2. Selye, H., *J. Clin. Endocrinol.*, 1946, v6, 117.
3. Selye, H., *Brit. M. J.*, 1950, v1, 203.
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8. Selye, H., and Pentz, I., *Canad. M.A.J.*, 1943, v49, 264.

TABLE I. Summary of Organ Weights.

Groups		I Normal controls	II STH	III DCA	IV STH+DCA
No. of rats per group		6	6	6	7
Initial body wt (g)		97	97	97	99
Final body wt (g)		126 ± 6.6	169 ± 6.4	111 ± 7.3	122 ± 10.2
Heart	mg	486 ± 35	664 ± 34	506 ± 25	620 ± 48
	mg/100 g	389 ± 26	392 ± 9.8	463 ± 33	511 ± 14
Kidney	mg	747 ± 54	1179 ± 113	940 ± 36	1447 ± 162
	mg/100 g	591 ± 14	693 ± 49	859 ± 51	1184 ± 96
Liver	mg	6313 ± 564	10681 ± 481	5953 ± 194	7720 ± 739
	mg/100 g	4975 ± 201	6331 ± 207	5450 ± 351	6322 ± 246
Adrenals	mg	30 ± 2.2	55 ± 2.1	24 ± 1.3	51 ± 4.2
	mg/100 g	23 ± 0.09	32 ± 1.1	22 ± 2.7	42 ± 2.3
Thymus	mg	208 ± 21	270 ± 13	134 ± 21	37 ± 9
	mg/100 g	164 ± 10	161 ± 11	121 ± 17	28 ± 4.8
Spleen	mg	1111 ± 54	2031 ± 188	965 ± 153	723 ± 140
	mg/100 g	893 ± 57	1211 ± 116	840 ± 100	566 ± 73

throughout the day and night, in order to obtain a relatively constant overdosage.

Desoxycorticosterone acetate was administered in the form of microcrystals as prepared by Merck and Co. (25 mg per cc), suspended in saline solution with added suspending agents. Of this 5 mg per day was injected subcutaneously once daily. Only the water intake was measured daily, as an index of *water turnover*, since the accurate collection of urine is technically difficult in the rat. In Table II the corresponding figures represent the mean values of the last seven days of observation.

Experimental results. The experiment had to be terminated after 14 days of treatment because all the animals receiving both STH and DCA (group IV) became extremely edematous and so weak that they obviously could not have survived much longer.

Changes in body and organ weights. In order to facilitate the evaluation of the changes observed, the weights of all organs have been expressed both in mg and in mg per 100 g of body weight with the standard errors.

In agreement with expectations, STH alone increased *somatic growth* very considerably. This weight increment was greatly counteracted by simultaneous administration of DCA, despite the manifest edema in group IV, which must have added considerably to the apparent weight of the rats. On the other hand, the enlargement of the *heart* and *kidneys* caused by STH was greatly augmented by simultaneous DCA treatment. The *liver* weight increase caused by STH was not further augmented by DCA. The "compensatory atrophy" of the *adrenals* caused by DCA was completely abolished by simultaneous STH treatment. Indeed, our STH proved to be strongly corticotrophic as judged by the adrenal weight increase, which it elicited when given either alone or in combination with DCA. Since this STH preparation was impure, its corticotrophic effect could have been due to contaminating ACTH; however, in our earlier work with electrophoretically pure STH this adrenal enlargement was also always very evident(5-7).

The *thymus* and *spleen* stimulating action of STH was actually diminished by simultaneous DCA administration, but this may be a non-specific effect due to the debilitated condition of the animals, with consequent endogenous ACTH liberation.

Histologic and clinical observations. The most important histologic and clinical observations are summarized in Table II.

Since in this experiment the animals were treated only during 2 weeks it is not unex-

5. Selye, H., *Brit. M. J.*, 1951, v1, 263.
6. Selye, H., *Am. J. Med.*, 1951, in press.
7. Selye, H., 2nd Clinical ACTH Conference, 1951, in press.

TABLE II. Summary of Some Histologic and Clinical Observations.

Groups	I Normal controls	II STH	III DCA	IV STH+DCA
Myocarditis	0	*	++	+++
Nephrosclerosis	0	+	+	+++
Pancreatic periarteritis	0	*	+	++
Water intake, cc	45 ± 1.6	93 ± 6.2	81 ± 3.9	163 ± 7.3
Subcut. and retroperitoneal edema	0	0	+	+++
Blood pressure	128 ± 3.0	148 ± 13	151 ± 12	129 ± 14

* Trace.

pected that neither STH nor DCA produced maximal histologic changes. Both these compounds, given singly, elicited the usual type of hyalinizing granulomatous myocarditis with periarteritis nodosa of the cardiac vessels, nephrosclerosis, inflammatory changes in the stroma and fat of the renal pelvis, proteinuria, and some extra-cardiac periarteritis nodosa, for instance in the pancreatic vessels, which are especially predisposed to this type of change. The water intake and diuresis was raised both by STH and by DCA alone, but only the latter produced a very mild degree of subcutaneous and retroperitoneal edema; even this was found only in some of the animals. On the other hand, conjoint treatment with STH and DCA resulted in a considerable potentiation of all these actions with marked myocarditis, nephrosclerosis and nephritis, pancreatic periarteritis nodosa, polyuria and edema formation. This marked sensitization of the cardiovascular and connective tissues by STH to the toxic effects of DCA may well account for the moribund condition of the animals in group IV. Both electrophoretically pure STH(5) and DCA(4) cause a rise in blood-pressure. This is confirmed by the present observations. The fact that conjoint treatment with the two hormone preparations did not result in any hypertension is pre-

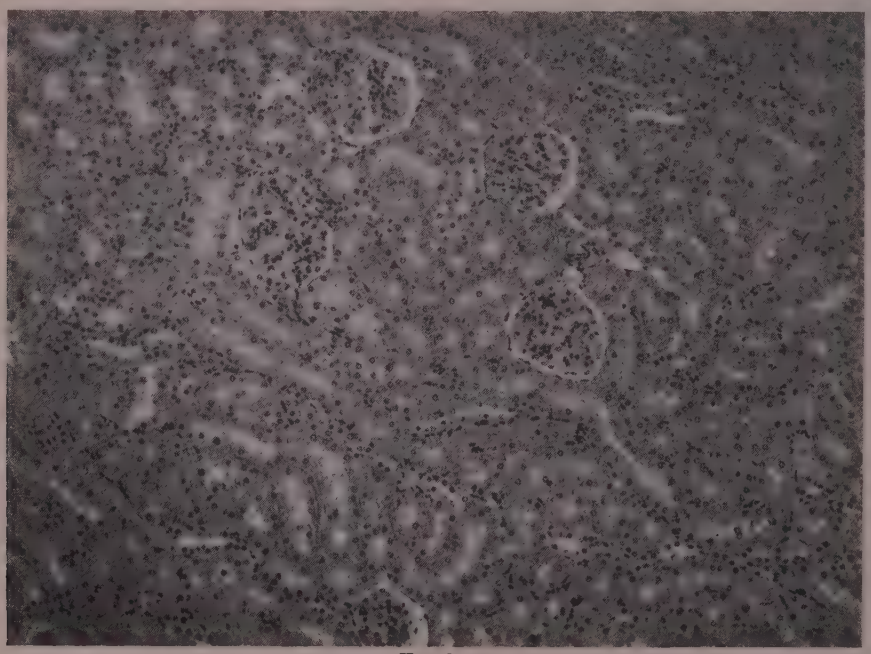

FIG. 1.
Kidney of normal control rat.

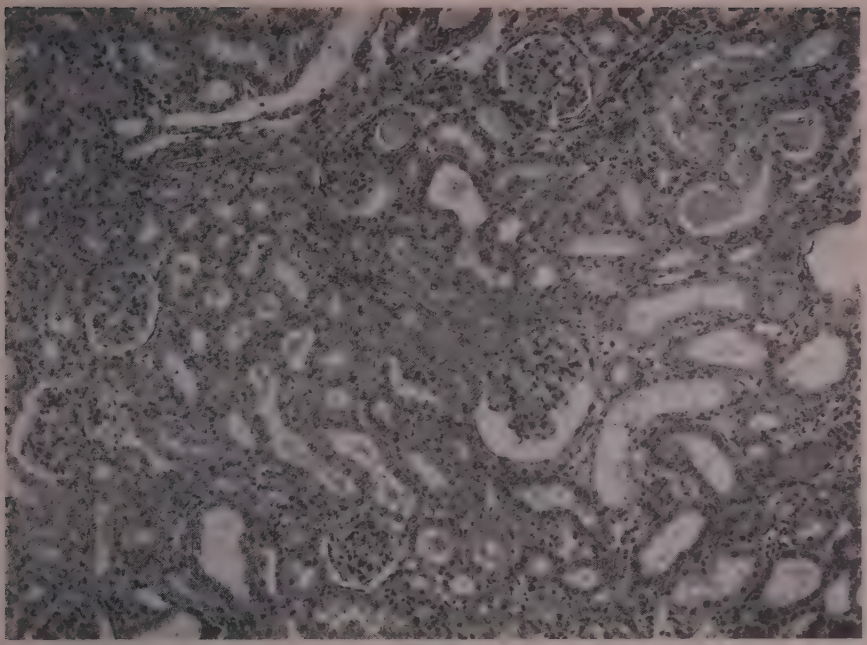

FIG. 2.

Kidney of STH-treated rat. Note dilatation of many tubules some of which contain hyalin casts or protein precipitates. The afferent arteriole of the central large glomerulus is almost completely hyalinized.

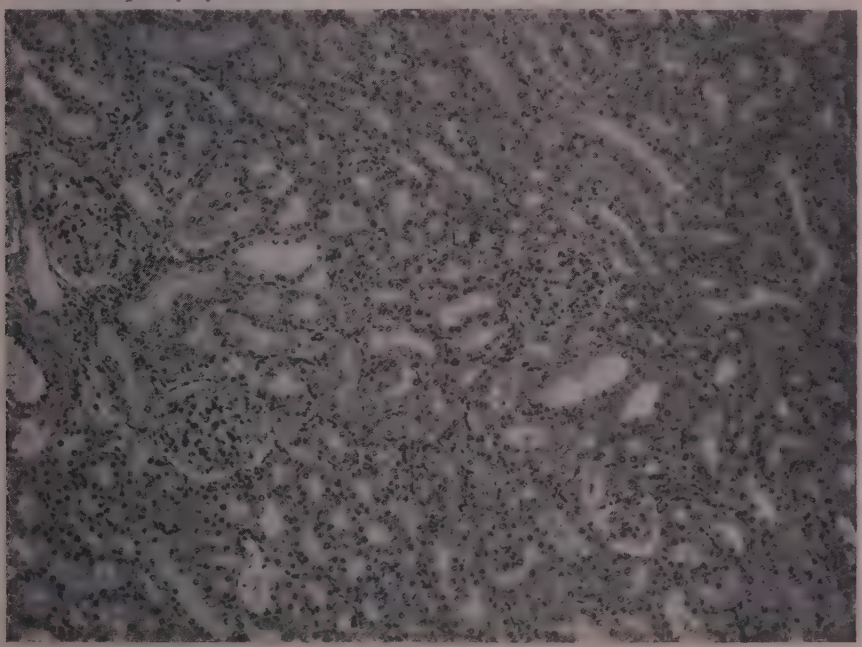

FIG. 3.

Kidney of DCA-treated rat. Note some tubular dilatation and protein precipitation, especially in the tubules situated in the center of the field. Surrounding this central region are several collapsed tubules exhibiting the appearance of "endocrine nephrons."

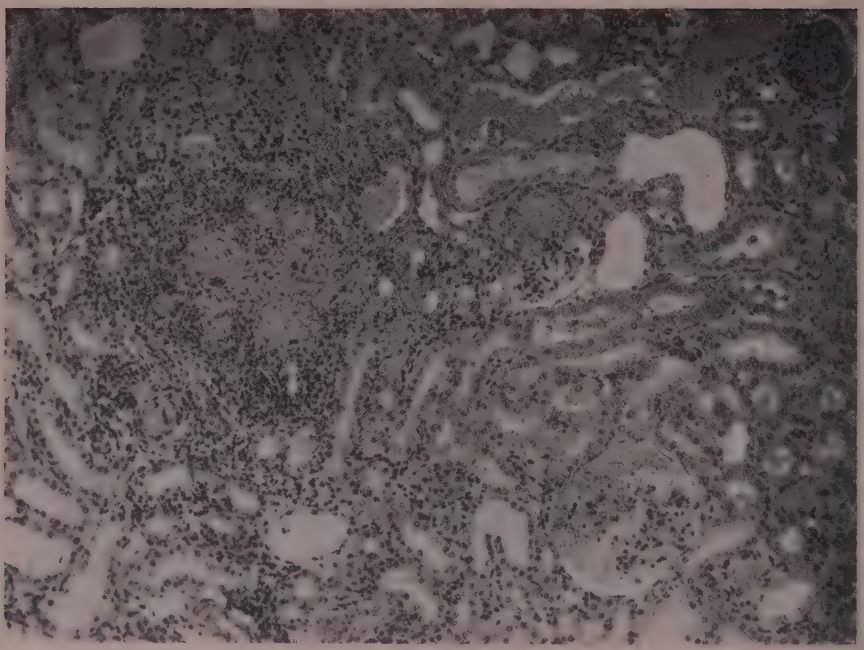

FIG. 4.

Kidney of rat treated with STH and DCA. Note one intensely hyalinized glomerulus in lower right corner of the field as well as several hyalinized blood vessels surrounded by an inflammatory granuloma in the upper left portion of the field. Most of the tubules are greatly dilated; some of them contain papillomatous excrescences, others proteinaceous material or hyalin casts.

sumably due to the poor physical condition of the animals.

Summary and conclusions. 1. Experiments on rats—sensitized to mineralo-corticoid actions by unilateral nephrectomy and a high sodium intake—showed that somatotrophic hormone (STH) shares with desoxycorticosterone acetate (DCA) the ability to produce: cardiac hypertrophy with histologic signs of a rheumatic-like myocarditis, hypertension, renal enlargement with microscopic evidence of malignant nephrosclerosis and an increased water turnover with proteinuria. 2. All these actions are greatly increased when STH and DCA are administered simultaneously, except for the rise in blood-pressure. The failure to develop hypertension under the influence of the two preparations may well be due to the fact that this hormone combination is extremely toxic and causes pronounced edema with obvious deterioration in the physical state of the animals. 3. It is concluded that STH sensitizes the tissues to the production of "collagen-disease-like" lesions by DCA. 4. STH also causes adrenocortical enlargement and prevents the compensatory atrophy of the adrenal cortex normally elicited by DCA. This effect may be due: to a contamination of our preparation with ACTH, to the compensatory secretion of ACTH by the pituitary of the STH-treated animal, or to a true corticotrophic action of STH. 5. Although earlier experiments had shown that even electrophoretically pure STH causes similar adrenal stimulation, we have no evidence either for or against the view that STH induces the adrenal cortex to produce mineralo-corticoids. The "mineralo-corticoid-like" actions of STH could result solely from the sensitization of the peripheral tissues by STH. Increased mineralo-corticoid production by the adrenal cortex under the influence of STH may be an additional factor, but its possible participation remains to be demonstrated.

These investigations have been performed with the help of the National Heart Institute (U. S. A.). The author is also indebted to Professor E. Dingemanse of Amsterdam and to Dr. M. Tausk of Organon Oss, for supplying the STH preparation, as well as to Miss Paulette Séguin and MM. Kai Nielsen and Robert Gagnon for technical assistance and the preparation of the micro-photographs.

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Electron Microscope Observations on Elastic Fibers. (18540)

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Elastic tissue generally considered both from chemical and submicroscopic analysis (1) as a relatively homogeneous entity, has been recently described as a two component system(2). Gross(2) in a very documented electron microscope analysis of elastic tissue from a variety of sources concluded that the elastic fibers are "composed of bundles of trypsin resistant threads of characteristic form and size plus a trypsin sensitive, heat-resistant 'amorphous' binding matrix." This conclusion was based on experiments in which digestion of either boiled or unheated elastic tissue with crystalline trypsin caused the "release" of characteristic thin coiled threads. These threads were observed in the surrounding of the partially digested fibers, but could not be observed with certainty within the fiber itself. In some recent experiments of digestion with crystalline trypsin on nerve material we were surprised to find threads which looked very similar to those described by Gross on elastic tissue. Furthermore, in control experiments in which the enzyme alone was incubated, similar threads could be observed, simultaneously with the presence of few bacteria and bacterial flagella.

These findings led us to reinvestigate the structure of the elastic tissue with the electron microscope, and to the demonstration that the threads described by Gross(2) are not elastic constituents.

Material and methods. The elastic material was obtained from the swimming bladder of the fish micropogon opercularis (Quoy and Gaimard). The preparation was similar to that described by Gross with minor modifications. The swimming bladder was cut into small pieces and stored overnight in 1% acetic acid at 4°C. The viscous mass obtained was diluted with more acetic acid and fragmented in a Waring Blendor. The elastin was separated from the ichthyocol (collagen) by repeated centrifugations and washing with dilute acetic acid. To complete the collagen extraction the suspension was boiled for 30 minutes and washed once more in acetic acid. The purified elastin was finally suspended in distilled water and boiled once more for sterilization. For this last operation glass beads had to be added to the suspension to overcome the strong tendency to stick of the purified elastin. Crystalline trypsin (Armour) dissolved to 0.1% in borate or phosphate buffer of pH 8 was used. Some digestion experiments were made under toluene, as described by Gross. In other cases a crystal of thymol was added to the fresh solution.

Digestion experiments under complete aseptic conditions were also performed. The trypsin solution was filtered through a Seitz microfilter. The buffer solution was sterilized by boiling and a final check of the pH value was made. Sterile glass material was used and all the operations were performed aseptically. The tubes containing the elastic material together with the controls were incubated at 37°-38°C for the same amount of time. After digestion the suspension was slightly disintegrated with a 10 Kc sonic magnetostriction oscillator. Specimens for electronmicroscopy

1. Wolpers, C., *Klin. Woch.*, 1944, v23, 169.
2. Gross, J., *J. Exp. Med.*, 1949, v89, 699.

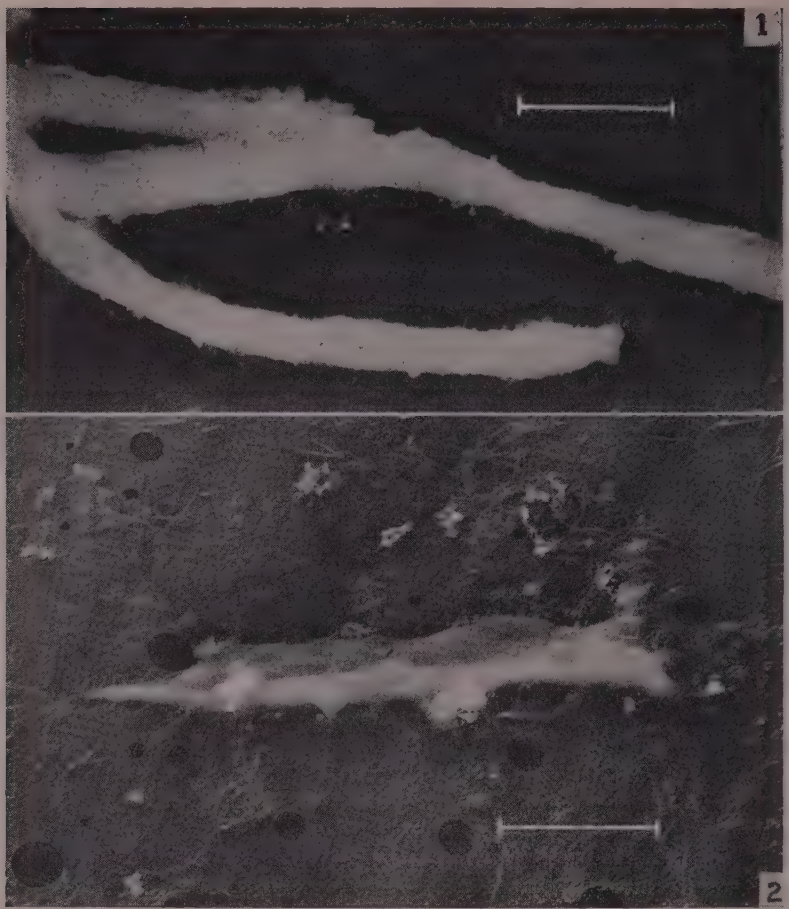

FIG. 1. Elastic fibers from fish swimming bladder. Longitudinal fibrillation within the fibers is apparent. Control material incubated for 6 hr in sterile buffer of pH 8. 22,000×.

FIG. 2. Elastic fiber partially digested with unfiltered trypsin. On the background and surrounding the fiber many thin threads are present. Incubation of 11 hr (with addition of thymol). 22,000×.

were prepared by depositing drops of the suspension on standard nickel grids. The preparations were shadowed with palladium at grazing angles of 9° to 11°. An R.C.A. type EMU 2C electron microscope was used and the microphotographs were generally taken at 6,000 X.

Results. Digestion of elastic material with unfiltered trypsin. The above described elastin suspension was added to the unfiltered trypsin solution in the proportion of 1 to 10 and was incubated for 11 hours. The results were similar to those obtained by Gross, *i.e.*, partially digested, flattened elastic fibers with moth-eaten edges and a coarse longitudinal fibrillation and on the background and surrounding the fibers the presence of a considerable number of fine short threads (Fig. 2).

The action of trypsin on the elastic fibers can be best appreciated by comparison with the control experiments in which sterile elastic suspension was incubated with buffer of pH 8. Characteristic long branching fibers flattened on the film and of variable diameter could be observed (Fig. 1). Usually they show a

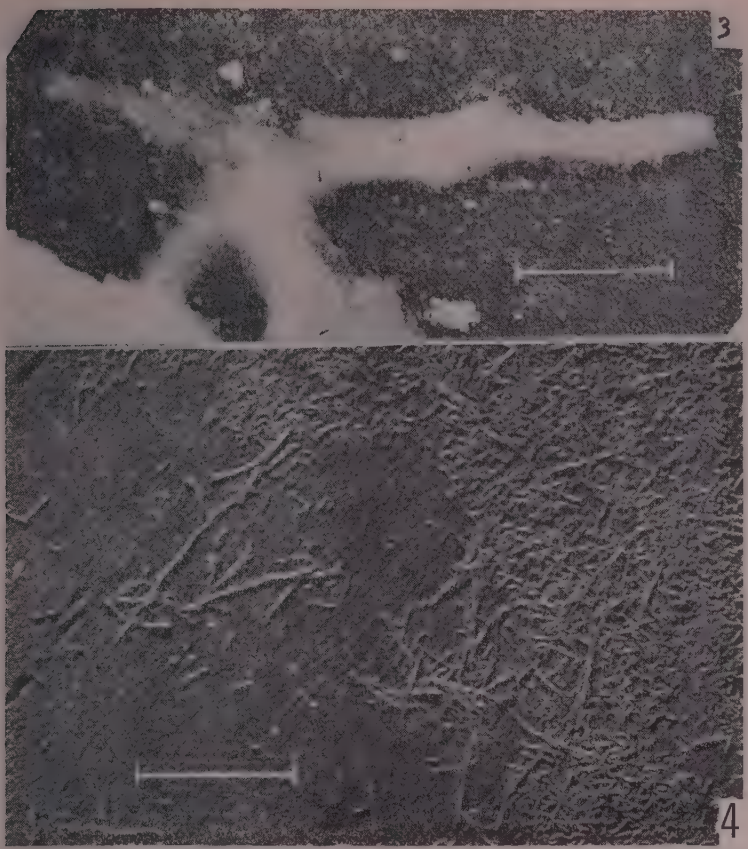

FIG. 3. Elastic fibers partially digested with filtered (sterile) trypsin. No threads are found. Incubation for 6 hr. 22,000×.

FIG. 4. Trypsin solution incubated for 11 hr (with addition of thymol). A considerable number of thin threads, similar to those found in Fig. 2 can be observed. 22,000×.

longitudinal fibrillation with tightly packed fibrils of about 400 to 700 Å in width. In these control experiments the background is completely clean, and threads are not found.

Digestion of elastic material with filtered trypsin. In this series of experiments complete aseptic conditions were insured as described above. Incubation periods ranged from 4 to 12 hours. Elastic fibers with different degrees of digestion were observed, but no threads could be found (Fig. 3).

Incubation of trypsin solutions under different conditions. In order to insure that the threads did not come from the elastic tissue the following control experiments were performed: (a) incubation of the trypsin solution (b) incubation of trypsin in sterile tubes (c) incubation of trypsin under toluene or with the addition of thymol (d) incubation of trypsin filtered through Seitz filter under aseptic conditions. All the tubes were incubated for 2 to 12 hours.

Samples of freshly prepared unfiltered trypsin solution were also examined. This fresh solution showed no threads, a few bacteria were present and constantly a finely granular and amorphous material was found which seemed characteristic for all dried samples of trypsin.

Tubes a, b, and c showed in all cases variable amounts of threads. A few bacterial bodies often showing attached flagella were

also found. Threads were of variable length and of about 120-150 Å in width (Fig. 4). A few of the threads showed a definite coiling.

Tubes with unfiltered trypsin stored in the refrigerator at 5°C for a period of a week also developed threads and many bacteria. In the experiment with filtered aseptic trypsin (tube d) neither threads nor bacteria were found even after long incubation periods.

Discussion. The experiments reported here clearly demonstrate that the "trypsin resistant threads" described by Gross as a characteristic component of the elastic fiber ultrastructure do not belong to this tissue. These threads appear in all cases in which incubation of the elastic tissue with trypsin is not done under absolutely sterile conditions. Neither toluene, as used by Gross, nor thymol are sufficient to prevent bacterial development and the appearance of thin threads. These threads have been also found by incubating other tissues with trypsin, and even trypsin alone, when complete aseptic precautions are not taken. These results lead to the conclusion that the thin coiled threads described by Gross belong to material digested from bacteria and probably from bacterial flagella. Experiments are under way in order to solve this problem.

Summary. Purified elastic material from fish swim bladder was fragmented and observed under the electron microscope. Elastic fibers were digested with unfiltered trypsin and also with trypsin sterilized by filtering through a Seitz microfilter. When digestion is not done under absolutely sterile conditions, "fine threads" described by Gross(2) as a component of the elastic tissue are observed.

If digestion is performed under complete sterile conditions no threads appear, and only partially digested elastic fibers can be found. Incubation of trypsin alone also shows the presence of threads when sterilization is not used and their absence in sterile conditions. The conclusion is reached that the threads described as a component of elastic tissue(2) belong to material digested from bacteria and probably from bacterial flagella.

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Experimental Amebic Hepatitis in Hamsters. (18541)

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While human infection with *Endamoeba histolytica* is most often manifested clinically as enteritis of varying severity, this pathogen frequently produces serious extra-intestinal lesions, particularly in the liver. Methods are available for producing experimental intestinal infections with *E. histolytica* in such animals as cats, dogs, rats, rabbits and guinea pigs. These infections under optimal conditions cause severe intestinal pathology with sufficient regularity to be useful in studies on intestinal amebiasis. However, such amebic infections are commonly restricted to the intestinal tract and hence are of very limited use for observations on extra-intestinal amebiasis. Cleveland and Sanders(1,2) were able to induce amebic hepatitis in cats by intrahepatic inoculation of amebae and associated bacteria but many early deaths, presumably from bacterial infection, and much variability in the incidence of hepatitis were encountered.

The foregoing considerations stimulated search for a more efficient experimental extra-intestinal amebic infection. When amebae were inoculated similarly into the liver of

* The authors are indebted to Dr. A. C. Bratton, Jr., and Dr. A. M. Moore for sustained interest in this work, and to Miss Anita Bayles, Mr. Robert Labay and Mr. James Ogg for technical assistance.

1. Cleveland, L. R., and Sanders, E. P., *Science*, 1930, v72, 149.

2. ———————— *Amer. J. Hyg.*, 1930, v12, 569.

small numbers of dogs, rats, mice, guinea pigs and hamsters, the most encouraging results occurred with the latter animal. The apparent superiority of the hamster from the standpoint of susceptibility to amebic hepatitis or of relative resistance to early death from bacterial infection lead to the studies which permit this preliminary report.

Experimental animals. Golden hamsters *(Cricetus auratus)* of either sex, weighing between 40 and 55 g, and approximately one month old were routinely used as experimental hosts. The animals were maintained in individual cages on a diet of Purina Laboratory Chow and fresh lettuce. During exploratory phases of the study differences in susceptibility did not appear to be associated with age. Statistical examination† of 188 subsequent, routinely infected animals revealed no evidence of sex influence on lesion size. Lesion weight tended to vary in proportion to initial body weight but the correlation was too weak ($\varrho = 0.18$, significant at 5% level) to be useful in refinement of lesion weight data in the narrow weight range employed.

Infecting organisms and infection procedures—Endamoeba histolytica of human origin, designated as strain 200 or BNIH(3-5), were maintained in culture at 37.5°C with 3 species of bacteria from the *coliform, Proteus* and *Streptococcus fecalis* groups, respectively, and hereafter referred to collectively as strain 200. Cleveland-Collier medium (Difco) was used with addition of a finely-milled preparation of Belgian rice starch (Stein-Hall Co.). The infecting inoculum, 25,000 or 50,000 amebae, from a 24-hour culture, was suspended in a menstruum of 0.05 ml of the culture medium overlay and infections were induced by direct injection into the liver following laparotomy under ether anesthesia. The operation site was prepared by cleansing with a germicidal agent, Phemerol Chloride.‡ A single injection was made into the ventral surface of the right hepatic lobe so as to produce a visible bleb beneath the hepatic capsule. Injections were made with a 27-gauge hypodermic needle with precautions to minimize seepage of the inoculum from the liver. A solution of Thrombin Topical (Parke, Davis and Co.) was applied to bleeding surfaces in an effort to control hemorrhage. The body wall was closed with skin clips, and the incision was covered with a solution of parlodion in ether.

Prevention of death from bacteria. When animals that had not been immunized against strain 200 bacteria were injected with the ameba-bacteria inoculum approximately 30% died within 72 hours. Heart blood cultures from animals dying during this interval revealed an overwhelming bacteremia, with *Proteus* as the predominant organism. It was found that intraperitoneal vaccination of animals, with living strain 200 bacteria, conferred virtually complete protection against the bacterial deaths following hepatic injection of amebae and bacteria. Immunization was then incorporated as part of the routine procedure and was conducted as follows: The bacterial component of the ameba strain was maintained free of amebae in Cleveland-Collier medium with added rice starch. The overlay from 24-hour cultures incubated at 37.5°C was diluted with an equal volume of sterile medium overlay, and 0.05 ml was injected intraperitoneally 6 days and 3 days before intrahepatic inoculation with amebae and bacteria. This immunizing procedure very rarely caused death, but approached the maximum tolerance of the animals since deaths were encountered in substantial numbers of animals when the total vaccine was given in a single injection.

Observations on the nature and etiology of the infections. Titration of the ameba-bacteria inoculum using 2-fold increments in numbers of amebae from 25,000 to 200,000, revealed no consistent relationship between the number of organisms and the size of the

† Dr. C. V. Winder.

3. Tobie, J. E., *Am. J. Trop. Med.*, 1949, v29, 859.

4. Thompson, P. E., and Lilligren, B. L., *Am. J. Trop. Med.*, 1949, v29, 323.

5. Thompson, P. E., Dunn, M. C., Bayles, A., and Reinertson, J. W., *Am. J. Trop. Med.*, 1950, v30, 203.

‡ Parke, Davis and Co. trademark for benzethonium chloride. Used here as a 1:1000 aqueous solution.

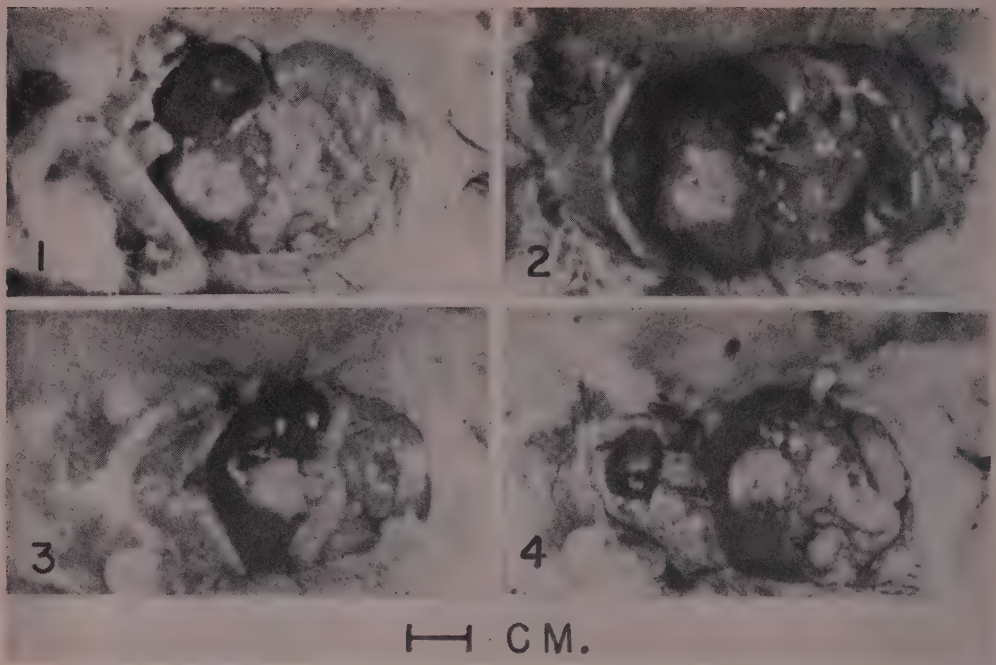

FIG. 1-4.
Amebic hepatitis in hamsters 92 hr after intrahepatic injection with 50,000 amebae.

lesions when animals were examined approximately 92 hours after infection. Greater variability in success of infection occurred with smaller numbers, although infections could be induced with as few as 3,000 amebae. Minimal variability occurred when the inoculum contained as many as 25,000 to 50,000 amebae and the latter has been selected as the usual infecting dose. Injections into the portal vein resulted in numerous small hepatic lesions, showing that trauma of the hepatic parenchyma was probably unnecessary for the routine infection. Successful inoculation into the liver, without appreciable seepage from the organ, produced usually a single well-defined lesion extending from the site of inoculation (Fig. 1-4), but occasionally metastasis to other lobes of the liver occurred. The lesion rarely liquefied but developed rapidly as a relatively homogeneous pale necrotic area until it involved by gross inspection from 25 to 50% of the liver. This amount of pathology appeared to be invariably fatal, with deaths occurring mainly between 6 and 8 days after infection. Amebae were readily demonstrable in the lesions by microscopic examination and by culture in Cleveland-Collier medium. An infection rate of approximately 95% has been observed among over 1000 hamsters inoculated during a period of 17 months. Hematogenous dispersal of amebae occurred commonly and was most readily apparent from the development of numerous discrete amebic lesions throughout the lungs.

The rate of lesion development is apparent from the data in Table I, which were collected during a single experiment. The animals were vaccinated against bacteria, injected intrahepatically with 50,000 amebae and groups selected at random were sacrificed at the specified intervals after inoculation. These data reveal an approximately linear rate of lesion growth.

Microscopic study of a few hematoxylin and eosin-stained sections of lesions 56 and 92 hours after infection revealed extensive necrosis of the parenchyma, manifested by disruption of the hepatic lobule architecture, and pale staining of the cytoplasm and nuclei

TABLE I. Data Illustrating the Rate of Hepatic Lesion Development in Hamsters.

Hr. after inoculation with amebae	Mean lesion wt in g ± stand. error	No. positive for amebae / No. inoculated
48	.30 ± .02	8/8
56	.78 ± .02	8/8
74	1.18 ± .03	10/10
92	1.78 ± .03	10/10
110	*2.29 ± .05	10/10

* 3 animals died between the 92nd and 110th hr and were not included in determining lesion wt.

of hepatic and connective tissue cells. Cellular infiltration was slight and consisted only of small aggregates of leucocytes with pyknotic nuclei.

The following observations suggest that amebae rather than bacteria are the major etiologic agents in producing hepatitis. (1) When strain 200 bacteria alone were injected into 28 hamsters, progressive hepatitis had not occurred when autopsied 92 hours after infection—14 had no lesions and the remaining 14 had lesions no larger than the original implantation. (2) Typical amebic hepatitis developed among all of 4 animals when the ameba-bacteria inoculum was pretreated for 3 hours with a mixture of sodium penicillin G (0.6 mg/ml) and dihydrostreptomycin sulfate (1 mg/ml) and the animals were treated with 0.6 mg/kg/day of Penicillin SR§ and 100 mg/kg/day of dihydrostreptomycin. No apparent effect on lesion development occurred when 10 animals were infected without pretreatment of the inoculum but were given these antibiotics in doses of 4.5 and 100 mg/kg/day, respectively. In both instances the antibiotics were given subcutaneously twice daily for 4 days, with the first dose 5 hours before inoculation. (3) The incidence and rate of lesion development were entirely comparable in animals effectively immunized against bacteria and in those not immunized. (4) Lesions were rarely purulent by either gross or microscopic examinations. In contrast to these observations bacteria persisted in the lesions and were readily demonstrable by culture. For this reason it would seem that bacteria cannot be completely exonerated until lesions have been produced with bacteria-free amebae.

§ Parke, Davis and Co. trademark for a mixture containing 3 parts procaine penicillin G and 1 part sodium penicillin G and having a potency of 1167 International units/mg.

Discussion. The procedures and materials described here seem to provide a convenient means for studying various aspects of extra-intestinal amebiasis. Chief emphasis so far has been to explore their usefulness in evaluating the action of drugs against amebic hepatitis. Chemotherapeutic data which will be reported elsewhere indicate that the clinically established drugs for this entity—emetine dihydrochloride and chloroquine diphosphate—are active in hamsters. In addition, it has been found that two clinically useful antimalarial drugs—quinacrine hydrochloride and amodiaquin (Camoquin‖)—have therapeutic action in amebic hepatitis in hamsters.

Summary. Progressive and usually fatal amebic hepatitis can be induced readily in golden hamsters by the intrahepatic injection of a strain of *Endamoeba histolytica* and its bacterial associates from cultures. Amebae rather than bacteria appear to constitute the major etiologic agents in producing the hepatitis.

‖ Parke, Davis and Co. trademark.

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Antibiotics and Hexamitiasis in Turkeys. (18542)

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Hexamitiasis, a protozoan disease of turkeys, of considerable economic importance, has been described by Hinshaw(1). Up to the time at which we began our studies June 2, 1950, there were no reports of any effective means of medication for the control of this endemic and frequently acute disease. A group of 9-week-old Broad Breasted Bronze poults, which had been reared in wire-floored batteries without contact with ground or other birds was employed. Several field cases of severe hexamitiasis were obtained and the hexamita collected in large numbers from the small intestine. The organisms were suspended in saline and equal doses of this suspension were introduced by pipette into the crop of each poult. In the following report, number of days indicated refers to time from this inoculation.

At the end of 5 days all birds appeared ill. Three were killed and found to have heavy infection of hexamita in the small intestine. The organisms were identified by microscopic study of smears from the small intestine(1). The remaining birds were treated as follows.

Control group. At the end of the 8th day this group appeared very ill. Three birds were killed for examination. All 3 showed heavy infection and a marked dehydration which is characteristic of this disease. The remaining 3 birds were fed a rearing mash containing 0.1% Enheptin* to the 12th day. One of these birds was then found to be free of detectable hexamita while 2 showed a very light infection. All were recovering from dehydration and appeared alert and vigorous.

Enheptin group. At 5 days, 6 of the diseased birds were given 0.1% Enheptin in mash. At 8 days 3 of these birds were found free of hexamita and noticeable dehydration. At 14 days the remaining 3 birds maintained on the mash with drug appeared to be in excellent condition and were free of hexamita. Nine days of continuous feeding of the drug did not appear to be harmful.

Aureomycin group. At 5 days, 6 diseased birds were fed aureomycin at 200 mg per lb of mash (.044%). At 8 days 3 of these birds were free of hexamita and signs of dehydration. The remaining birds were kept on the drugged mash to 14 days, when they also were found free of the organisms and were in excellent condition.

Streptomycin group. At 5 days, 6 diseased birds were fed streptomycin in 25 mg capsules, 1 per day for 3 days. At 8 days, 3 birds showed remaining heavy infection with hexamita and distinct dehydration. One bird died overnight and was not examined. After the 8th day 2 birds were fed 0.1% Enheptin in the mash. At 12 days these birds were free of hexamita and signs of dehydration.

Penicillin group. At 5 days, 6 diseased birds were given penicillin-G by 25 mg capsule, 1 per day for 3 days. At 8 days, 3 birds were free of hexamita and only slightly dehydrated. The remaining birds were kept to 14 days with no treatment. At this time these birds showed no evidence of hexamita or dehydration.

Terramycin group. At 5 days, 6 diseased birds were given terramycin by 25 mg capsule 1 per day for 3 days. At 8 days, 3 birds showed no evidence of hexamita or dehydration. The remaining birds were kept to 14 days without further treatment. At this time one was free of the disease and two showed very few hexamita organisms.

The results of this work, although preliminary in nature, strongly suggest that it may be possible to control hexamitiasis in turkeys by certain drugs or antibiotics. Four such drugs appeared beneficial, while one was not. The evaluation of most effective drugs and manner of administration remains to be determined.

1. Hinshaw, W. R., *Univ. Calif. Agr. Exp. Sta.*, Bul., 1943, v613, 97.

* 2-amino-5-nitrothiazole. Lederle Laboratories, Inc., Pearl River, N. Y.

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Protective Action of Certain Amino Acids on Toxicity of Mercurial Diuretics in Rats. (18543)

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Mercurial diuretics given in therapeutic doses with the intention of inducing diuresis rarely cause renal damage. Comparatively larger doses, however, will regularly produce severe renal injury in the experimental animal. Lippman(1) has shown that the administration of human albumin to rats prior to the administration of mercurial diuretics sharply reduced renal toxicity in the rat. It had been previously demonstrated that the severe necrotizing nephrosis induced by dl-serine in the rat can be favorably influenced by several amino acids and related compounds(2). In the present study a considerable protective action of various amino acids was established against the nephrotoxic effect of a mercurial diuretic.

Methods. More than 300 young female albino rats weighing approximately 150 g were used. The mercurial diuretic employed was Meralluride Sodium ("Mercuhydrin Sodium," Lakeside Laboratories Inc., Milwaukee, Wisc.). This preparation contains 39.0 mg mercury and 48 mg theophylline in 1 ml. The rats were given 0.01 mg mercury per g of body weight or approximately 2 ml of a 1 to 50 dilution of the original preparation intramuscularly. It was established in preliminary experiments that this amount produced regularly severe necrotizing nephrosis within 24 hours. The amino acids to be tested were dissolved in distilled water, adjusted to a pH ±7.2 if necessary and injected in amounts of 3 ml intraperitoneally 30 minutes before, simultaneously with, and 30 minutes after the mercurial was given. In some experiments (see Table I) a different schedule was used. The animals were killed after 24 hours and the kidneys fixed in formalin. Sections for microscopic study were stained with hematoxylin and eosin. Ten to 12 animals were used at one time. At least 2 animals served as controls. These were given the mercurial intramuscularly and corresponding amounts of physiological saline solutions intraperitoneally. The degree of renal damage was expressed as 0, ±1+, 2+, 3+, and 4+. In kidneys considered to show ± damage only very few necrotic tubules could be seen while in those with 1+ damage occasional ones appeared necrotic. The degree of protection given by a certain compound was judged by the percentage of animals which showed 0, ±, and 1+ lesions in this particular group. In order to further evaluate the degree of the renal injury, urinary protein excretion was measured. For this purpose rats were put in individual metabolism cages and 24 hour urine specimens collected. Protein was determined with Exton's method using a Leitz photocolorimeter(3). After protein excretion had been examined for 5 consecutive days, one group of 17 rats was injected with 1(+) arginine monohydrochloride and the mercury preparation while another group of 15 animals were injected with the mercurial only. Urine was collected for the following 7 days and the animals then killed and their kidneys prepared for microscopic study.

Results. Control animals. Severe necrotizing nephrosis restricted to the proximal convoluted tubules was regularly seen within 24 hours on microscopic sections (see Fig. 1). The appearance was identical with that observed following the application of mercury bichloride. With the amount of mercurial given only 1 out of 57 animals tested showed a 1+ lesion, while the damage of the kidneys of the remaining animals was graded from 2+ to 4+, with 42 rats revealing a 4+ involvement (Table II).

In 15 rats killed after 7 days there was the

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2. Wachstein, M., *Arch. Path.*, 1947, v43, 515.

3. Exton, W. G., *J. Lab. and Clin. Med.*, 1925, v10, 9.

TABLE I. Summary of Results of Experiments to Determine Whether 1 or 2 Injections of 1(+) Arginine Monohydrochloride Given at Various Time Intervals Would Protect Rats Against Renal Injury Caused by Merullaride Sodium.

30 min. before	Simult.	30 min. after	60 min. after	90 min. after	Total No. rats	No. of rats showing lesions 0	±	1+	2+	3+	4+	Rats showing 0, ±, and 1+ lesions No.	%
					One injection								
+	—	—	—	—	6	0	1	1	2	1	1	2	33.3
—	+	—	—	—	6	0	3	0	2	1	0	3	50
—	—	+	—	—	6	0	0	4	2	0	0	4	66.6
—	—	—	+	—	6	0	1	0	3	2	0	5	20
					Two injections								
+	+	—	—	—	6	0	3	1	1	1	0	4	66.6
—	+	+	—	—	6	0	3	1	1	1	0	4	66.6
—	—	+	+	—	12	1	5	3	1	2	0	9	75
—	—	—	+	+	6	0	0	1	1	1	3	1	20

typical dilatation of many proximal convoluted tubules which were invested by flat regenerating epithelium. Calcification in damaged tubules was occasionally seen.

Influence of various amino acids given in 3 divided doses. The substances tested can be seen in Table II. Among the amino acids used 1(+) arginine monohydrochloride and glycine gave very considerable protection when three times 300 mg were administered. dl-alpha-alanine and 1(+) histidine monohydrochloride follow in their protective potency, while dl-methionine and 1(+) cysteine hydrochloride were only of moderate influence. With half the amounts given 1(+) arginine monohydrochloride was still quite effective while dl-alpha-alanine and glycine had only little effect. No protective action was seen from dl-threonine, dl-valine, dl-phenyl-alanine, dextrose and sodium chloride.

FIG. 1.
Section of kidney of a rat 24 hr after meralluride sodium (.01 mg mercury per g body wt). The renal damage is considered to be 4+. Hematoxylin-Eosin stain ×60.

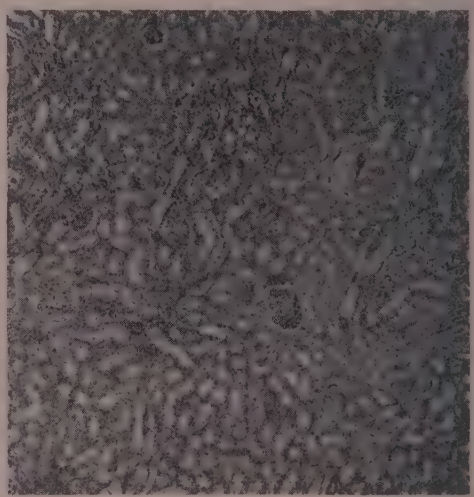

FIG. 2.
Section of kidney of a rat 24 hr after meralluride sodium (.01 mg mercury per g body wt) and protected by 3 inj. of 300 mg 1(+) arginine monohydrochloride. There is no renal damage seen. Hematoxylin-Eosin stain ×60.

Influence of 1 or 2 doses of 1(+) arginine monohydrochloride given at various time intervals. Table I shows the effect of 1 or 2 injections of 300 mg 1(+) arginine monohydrochloride upon the mercurial toxicity. Two injections given 30 minutes prior and

TABLE II. Summary of Results of Experiments to Determine Whether Various Amino Acids Protect Rats Against Renal Injury Caused by Merullaride Sodium.

Substance tested	Amt in mg, 3×	Total No. rats	No. of rats showing lesions 0	±	1	2	3	4	Rats showing 0, ±, and 1+ lesions No.	%
—	—	57	0	0	1	8	6	42	1	1.8
l(+) arginine monohydrochloride	300	19	3	10	5	1	0	0	18	94.7
l(+) arginine monohydrochloride	150	16	0	7	2	5	2	0	9	56.3
Glycine	300	13	1	9	2	1	0	0	12	92.3
"	150	8	0	0	1	4	2	1	1	12.5
DL-alpha-alanine	300	24	0	11	5	4	4	0	16	66.6
"	150	9	0	1	1	3	3	1	2	22.2
l(+) histidine monohydrochloride	300	6	0	3	1	0	1	2	4	66.6
l(+) cysteine hydrochloride	300	9	3	1	0	5	0	0	4	44.4
DL-methionine	150	8	0	1	2	0	2	3	3	37.5
DL-threonine	300	7	0	0	0	0	0	7	0	0
DL-valine	150	4	0	0	0	0	0	4	0	0
DL-phenylalanine	75	3	0	0	0	0	1	2	0	0
Dextrose	300	6	0	0	0	0	0	6	0	0
Sodium chloride	150	8	0	0	0	2	3	3	0	0

simultaneously with the drug or simultaneously and 30 minutes later, or 30 and 60 minutes later imparted considerable protection. Only little influence was seen if the two injections were given 60 and 90 minutes later. In the case of one injection an appreciable protective influence was demonstrable if the amino acid was administered simultaneously or 30 minutes after the mercurial had been given.

Proteinuria in animals protected and unprotected by l(+) arginine monohydrochloride. In agreement with the findings of others there was considerable individual variation in the amounts of protein excreted in the urine under normal conditions(4,5). In 32 rats the 24 hour excretions of protein averaged 3.7 mg and the average urine quantity 8 ml. Following the injection of mercuhydrin urinary protein excretion rose sharply (Fig. 3); the main increase took place in the first 72 hours following injection. The average excretion for 15 rats in this period amounted to 91.2 mg while the excretion of protein in 17 rats protected by 3 injections of l(+) arginine monohydrochloride was only 42.1 mg. The diuretic effect of mercuhydrin in both groups was not significantly different (Fig. 4).

On microscopic examination the degree of renal damage in the control group was con-

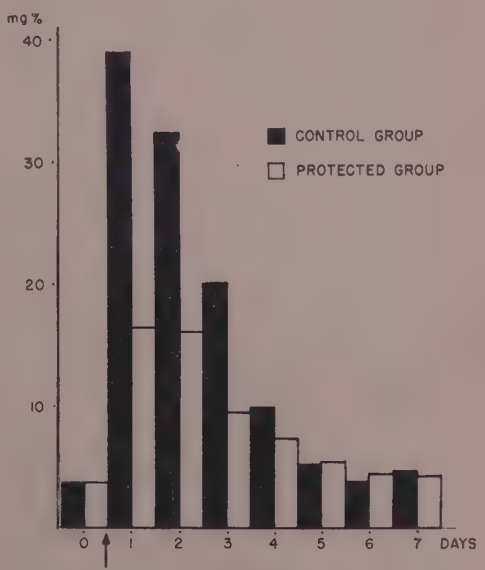

FIG. 3.

Average 24 hr urinary excretion of protein in a group of 15 rats before and following a single inj. of meralluride sodium (.01 mg mercury per g body wt) (dark column) and of a group of 17 rats protected with 3 inj. of 300 mg l(+) arginine monohydrochloride (white column).

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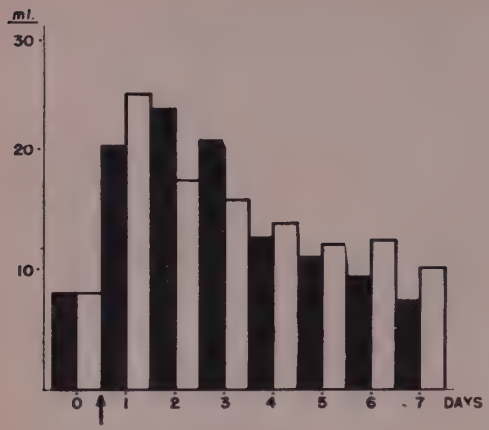

FIG. 4.

Average 24 hr excretion of urine in group of 15 rats before and following a single inj. of meralluride sodium (.01 mg mercury per g body wt) (dark column) and of 17 rats protected with 3 inj. of 300 mg 1(+) arginine monohydrochloride (white column).

sidered to be 1+ in one, 2+ in 3, 3+ in 4 and 4+ in 7 rats. There was therefore severe involvement in 93.4%. Among the 17 rats injected with 1(+) arginine monohydrochloride no renal damage was seen in 3. In the remaining 14 animals the lesion was graded as ± in 4, as 1+ in 2, as 2+ in 3, as 3+ in 4 and as 4+ in 1. Marked renal involvement was therefore found in 8 animals or 47%.

Comment. It is generally agreed that mercurial diuretics act by depressing tubular function and impair the reabsorption of sodium and water. In man they depress the reabsorption of glucose(6) as well as other functions ascribed to involve the proximal convoluted tubules(7). However, in the dog the tubular transfer of glucose and p-aminohippurate is not influenced by mercurials (8,9). The depression of tubular reabsorption of sodium is furthermore limited in the dog. According to Duggan and Pitts(9) this limitation is conditioned by the magnitude of the absorptive system which corresponds to the magnitude of the distal tubular absorption. The microscopic changes regularly seen with toxic doses in the rat are identical with those observed following mercury bichloride. With special technics the renal damage has been localized under the latter condition to the distal portion of the proximal convoluted tubules in the dog(10) and the median portion of the proximal convoluted tubules in the rat(11). The *microscopically* demonstrable influence of mercuhydrin sodium upon the renal cortex is therefore seen only in the proximal convolution even in highly toxic doses.

The experiments reported here show conclusively that certain amino acids given in large amounts are able to modify the nephrotoxic action of a mercurial diuretic. A similar protective effect of some amino acids has been established previously against the kidney damaging action of dl-serine in the rat(2). It was assumed that the beneficial effect of these amino acids was due to their competitive suppression of tubular reabsorption of the injurious d-isomer of the dl-serine. Competition for transport has not only been reported for amino acids but also for a number of compounds unrelated by chemical structure(12). The protective effect of human albumin upon the renal toxicity of mercurial diuretics was thus attributed by Lippman(1) in a similar manner to the inhibition of mercurial reabsorption when the tubules are saturated with protein. In those experiments no protection occurred if the protein was given simultaneously with the drug. In contrast 1(+) arginine monohydrochloride was found quite effective if given simultaneously or even 30 minutes later. This difference can be explained by the more rapid absorption of amino acids due to their smaller molecular size. There is a marked difference in the protective effect of certain amino acids against the toxic effect of dl-serine and that of mercuhydrin

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11. Edwards, J. G., *Am. J. Path.*, 1942, v18, 1011.

12. Taggart, J. V., *Am. J. Med.*, 1950, v9, 678.

sodium. dl-threonine for instance was found to protect the kidney completely against dl-serine but is without effect on the toxicity of mercuhydrin sodium. These differences are at the present time unexplained.

Summary. Certain amino acids reduce considerably the nephrotoxic action of meralluride sodium in the rat. Among the amino acids examined 1(+) arginine monohydrochloride and glycine were found to be most effective. The beneficial influence seen in microscopic sections as well as in the reduced proteinuria following a toxic dose of the mercurial diuretic is attributed to the competitive suppression of tubular reabsorption of the injurious mercury.

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Independent Biosynthesis of Different Hemin Chromoproteins—Cytochrome *c* in Various Tissues.* (18544)

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Since the same prosthetic group, iron protoporphyrin IX (structural type III porphyrin), is present in the different chromoproteins, hemoglobin, myoglobin, cytochrome *c* and catalase, it becomes of fundamental interest and importance to know whether the biosynthesis of the metalloporphyrin (protohemin) is confined to a localized site or whether porphyrin and hemin synthetic ability is a general property of cells of different tissues. Actually, very little information of decisive character is available as to the biosynthesis of chromoproteins other than hemoglobin. Experiments by Beinert *et al.*(1,2) on the incorporation of the radioactive iron isotopes in cytochrome *c* proved merely that if sufficient iron was supplied it would appear also in the prosthetic group of the widely distributed tissue pigment, cytochrome *c*, but left unsettled the question of relative degrees or rates of incorporation in the different chromoproteins, as the labelled iron was furnished in large doses during active growth from birth on, yielding rather similar very high levels of incorporation in all isolated pigment samples. On the other hand, in Theorell's laboratory, it has been shown with radioactive iron that the rate of incorporation of the iron in the hemin of liver catalase is significantly greater than in that of red cell catalase, from which it was deduced that the biosynthesis of the catalases of these respective tissues may be independent.‡ Work in our laboratory on the appearance of "new" cytochrome *c* in liver regenerating after partial hepatectomy(3-5) favored the inference that this chromoprotein was fabricated in tissues *in situ*, but the experiments were indecisive since the possibility of the mobilization of cytochrome *c*, its transfer to regenerating liver from tissues such as skeletal muscle, was not eliminated. A stronger deductive inference of the independent biosynthesis of cytochrome *c* could be drawn from the rapid changes in the cytochrome *c* content of various tissues after thyroidectomy and induced hyperthyroidism(6).

* This work has been done under contract between the Office of Naval Research and the University of Pennsylvania.

† With the technical assistance of Anna May Dych, Jean Randall, Charlotte Glausser and Pauline A. Corneille.

‡ Personal communication from Professor Hugo Theorell, Director of the Biochemical Department, Medical Nobel Institute.

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2. Beinert, H., Matthews, P. and Richey, E. O., *J. Biol. Chem.*, 1950, v186, 167.

3. Crandall, M., and Drabkin, D. L., *J. Biol. Chem.*, 1946, v166, 653.

4. Drabkin, D. L., *J. Biol. Chem.*, 1947, v171, 395.

5. Drabkin, D. L., *J. Biol. Chem.*, 1950, v182, 317.

TABLE I. Radioactivity of Cytochrome *c* (*C*) and Hemoglobin (*H*) After Parenteral Administration of Glycine-2-C^{14}.*

Rat	Days after partial hepatectomy	No. of doses of isotope*	Counts/min./mg†				
			Liver *C*	Skeletal muscle *C*	Kidney *C*	Heart *C*	Blood *H*
1 (control)		8	119‡	196	428§		118§
2 (control)		8	715	404			
3	11	8	1039	108	591	47	188
4	6	5	2850	192	474		

* See the text.

† Measurements were made with a windowless counter, and the counts were corrected for self-absorption. The counts per mM (1 iron atom equivalent wt) may be obtained by multiplying the cytochrome *c* counts per mg by 13000 and the hemoglobin counts by 16700.

‡ This result may be low owing to technical difficulties in the preparation of the sample.

§ Pooled sample from rats 1 and 2.

The present report is upon chromoprotein labelling obtained after the administration of glycine-2-C^{14}, and the interpretation of the findings.

Experimental procedure. Adult albino rats of 200 g body weight on a high protein diet (3,4) were used. Liver lobectomy in which 68 per cent of the liver was excised was performed as previously described(4). The glycine-2-C^{14} was administered intraperitoneally in similar amounts to intact controls and to the operated animals in equal divided doses over a period of 5 to 8 days. Each dose was 0.5 ml of aqueous glycine, containing 0.5 mg of the amino acid, with 5 μc of original activity (108,000 counts per minute per μc).

Cytochrome *c* was isolated by the method of Rosenthal and Drabkin(7) and the quantity was determined by our direct micro spectrophotometric technic(7). The isolated material was diluted 50- to 100-fold with highly pure non-radioactive cytochrome *c* (analysis = 0.414% iron; theory = 0.43)§ as carrier, and the mixture dissolved and then resubjected (for purification)(8,9) to the steps of the isolation procedure, including thorough washing with non-radioactive glycine solution to minimize "quasi-incorporation." The cytochrome *c* was finally plated for counting by precipitating it with acetone. The hemoglobin from thoroughly washed red cells was also precipitated with acetone. The precipitate was washed with inactive glycine, and plated from acetone suspension.

Results. Table I is a summary of data on the radioactivity of samples of cytochrome *c* from various tissues and of hemoglobin, after the administration of glycine-2-C^{14}. Attention is directed to the following: (1) The carbon of glycine-2-C^{14} was actively incorporated in the cytochrome *c* of tissues. (2) Appreciably higher activity was obtained in the cytochrome *c* of regenerating liver than in that of the control liver. (3) Muscle cytochrome *c* had a much lower specific activity, and, hence could not be the source of liver cytochrome *c*, unless the unlikely assumption be made that muscle contained more than one species of cytochrome *c*, one of which was highly active (labelled) and preferentially migrated from the muscle. (4) The degree of labelling of the cytochrome *c* of different tissues was different. This could be partially due to the dilution effect of the total cytochrome *c* content, as in the case of skeletal muscle, which contains about 77% of the total cytochrome *c* of the body(3-5), but hardly explains the unusually low incorporation of C^{14} in the cytochrome *c* of heart. This very interesting finding in a tissue of small relative mass, with a high concentration level of cytochrome *c* and high oxidative metabolism(3,5), is unfortunately limited to this isolated instance, and will be subjected to further investigation. It is suggestive of a lower "turn-

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7. Rosenthal, O., and Drabkin, D. L., *J. Biol. Chem.*, 1943, v149, 437.

§ Kindly supplied by the Wyeth Institute of Applied Biology (cf. 9).

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9. Tint, H., and Reiss, W., *J. Biol. Chem.*, 1950, v182, 385 and 397.

TABLE II. Comparison of C^{14} Incorporation in Liver Cytochrome *c* and Blood Hemoglobin and Provisional "Turnover" Rates.*

Chromoprotein	Total in organ, mg	Total "new" chromoprotein, mg	Total "old" chromoprotein, mg	Mean activity of total chromoprotein counts/min./mg	Calculated activity of "new" chromoprotein counts/min./mg	Calculated "turnover" (provisional) %/day
Liver cytochrome *c*						
Control	1.450†			715		
Regenerating 6 days	1.323‡	.868‡	.455‡	2850	3970§	
Regenerating 11 days	1.323‡	.868‡	.455‡	1039		12.7‖
Blood hemoglobin	3190†	287¶		188	2090	0.8¶

* See the text.

† Based on Crandall and Drabkin(3).

‡ Based on Drabkin(4,5).

§ Total activity of "old" liver cytochrome $c = 715 \times 0.455 = 325$; activity of 0.868 mg of "new" cytochrome $c = (2850 \times 1.323) - 325 = 3445$; activity per mg of "new" cytochrome $c = 3445/0.868 = 3970$.

‖ Mg of unlabelled cytochrome *c* necessary to replace liver cytochrome *c* to reduce count of 2850 to 1039 in 5 days $= ((2850-1039)/2850 \times 1.323 = 0.841$ mg. Rate of "turnover" $= 0.841/5 = 0.168$ mg of liver cytochrome *c* per day, or $(.168 \times 100)/1.323 = 12.7\%$ per day.

¶ Based on life span of approximately 120 days for mammalian erythrocytes. With a "turnover" of 0.8% per day, 9% of the circulating mature red cell population would be labelled during 11 days.

over" of cytochrome *c* in cardiac muscle.

In Table II the radioactivity data are recalculated for liver cytochrome *c* and blood hemoglobin so as to secure a sounder comparison of the relative degrees of isotopic labelling, under our conditions, of the two chromoproteins. The calculations are explained in the footnotes to the table. It may be seen that after adjustment to the highly different dilution spaces of 3190 mg of total hemoglobin and 1.32 mg of liver cytochrome *c*(3-5), the labelling of "new" cytochrome *c* in liver is only 2-fold greater than that of hemoglobin. Since nearly all of the "new" cytochrome *c* of liver is laid down by the sixth day of the regenerative process, and the quantity of the pigment then remains relatively constant(4), a provisional rate of "turnover" of liver cytochrome *c* may be calculated from the values of activity at 6 and 11 days following partial hepatectomy. The "turnover" value calculated from the data is 12.7 per cent per day, which is appreciably greater than the normal daily "turnover" of hemoglobin of approximately 0.8%(10). It is of interest that the "turnover" deduced by this method is of the same magnitude as that previously calculated by us from the rate of appearance of "new" cytochrome during active liver regeneration (4).

Discussion. It has previously been established that glycine is an important intermediary metabolite in the biosynthesis of hemoglobin(11). The present finding of active incorporation of C^{14} from glycine-2-C^{14} into cytochrome *c* suggests that this amino acid probably participates in chromoprotein biosynthesis in general. However, it should be pointed out that the use of a common precursor does not preclude independence of hemin and chromoprotein biosynthesis in the sense of the capability of different tissues of doing the synthetic job. The possibility that muscle cytochrome *c* can migrate to liver and account for the increase in "new" cytochrome *c* during liver regeneration has been excluded by the present findings. Also, the data lend strong support to the view that liver cytochrome *c* is produced *in situ* and independently of the cytochrome *c* of other tissues and hemoglobin. Parenterally injected cytochrome *c*(12) and labelled cytochrome *c* containing radioactive iron(2) are not incorporated in tissues.

The present experiments do not serve to

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12. Drabkin, D. L., *J. Biol. Chem.*, 1947, v171, 409.

exclude the possibility that liver cytochrome *c* may move to other tissues, nor do they exclude the possibility for movement from tissue to tissue of mobilizable smaller molecule precursors.

The "turnover" rate of liver cytochrome *c* is regarded as provisional, since at present we possess no information on the glycine pool in localized areas of the body. Degradation of the labelled chromoproteins, now in progress, will reveal whether the ratio of C^{14} incorporation in hemin and in the protein moiety is different for the cytochrome *c* of different tissues and for hemoglobin. It is also desirable to know whether the metabolism of cytochrome *c* in tissues is dynamic, or "static" as in the case of hemoglobin in mature red cells.

Summary. The participation of glycine in the biosynthesis of cytochrome *c* has been demonstrated. Under our conditions, the incorporation of C^{14} from glycine-2-C^{14} was of relatively very low order in the cytochrome *c* of heart and very high order in liver tissue which had undergone active regeneration. The data support the conclusion that in regenerating liver cytochrome *c* is fabricated *in situ* and not derived from other tissues. A rate of "turnover" for liver cytochrome *c* has been provisionally deduced, which is appreciably higher than the normal rate of hemoglobin "turnover." It is tentatively proposed that chromoprotein biosynthesis is a general property of living, aerobic cells.

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Liver Injury Following Administration of α- and β-Longilobine.* (18545)

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The *Senecio* species first attracted attention when they were found to be responsible for the death of cattle and horses in Canada, South Africa, New Zealand, and the United States. Cushny(2) described the effects of 2 alkaloids, senecifolin and senecifolidine, both isolated from *Senecio latifolius,* in dogs, cats and rats. Since that time some 40 alkaloids of *Senecio* have been studied. One of these is longilobine which occurs in *Senecio longilobus,* a plant indigenous to western Texas, and responsible for great cattle losses. Clawson(3) showed *Senecio longilobus* to be one of the more poisonous of the American species of *Senecio.* Horses, sheep and cattle succumbing to a diet of this plant were found to suffer from extensive liver damage. Longilobine was isolated by Manske(4). Adams and Govindachari(5) employing a chromatographic procedure were able to separate longilobine into two components, α- and β-longilobine. Chemical investigation by Warren and his associates(6) proved that β-longilobine is identical in structure with retrorsine.

Previous studies in this laboratory(7-9) showed that Manske's longilobine and retrorsine caused liver damage in white mice follow-

* Presented in part at the Fall meeting of the American Society for Pharmacology and Experimental Therapeutics, Indianapolis, 1949(1).

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2. Cushny, A. R., *J. Pharm. and Exp. Therap.,* 1910-11, v2, 531.

3. Clawson, A. B., *Veterinary Medicine,* 1933, v28, 105.

4. Manske, R. H. F., *Can. J. Res.,* 1939, v17B, 1.

5. Adams, R., and Govindachari, T. R., *J. Am. Chem. Soc.,* 1949, v71, 1180.

6. Warren, F. L., Kropman, M., Adams, R., Govindachari, T. R., and Looker, J. H., *J. Am. Chem. Soc.,* 1950, v72, 1421.

7. Harris, P. N., Anderson, R. C., and Chen, K. K., *Am. J. Physiol.,* 1941, v133, 318.

8. Harris, P. N., Anderson, R. C., and Chen, K. K., *J. Pharm. and Exp. Therap.,* 1942, v75, 69.

9. Chen, K. K., Chen, A. L., and Rose, C. L., *J. Pharm. and Exper. Therap.,* 1935, v54, 299.

ing intravenous injection. No pharmacological information is as yet available in regard to the relative activity of the 4 closely related alkaloids. Professor Roger Adams, Department of Chemistry, University of Illinois, Urbana, kindly supplied us with α- and β-longilobine. The object of the present work was to make a direct comparison of these two alkaloids with Manske's longilobine and retrorsine in albino mice.

One per cent solutions of longilobine, α- and β-longilobine were prepared by neutralizing the required amount of each base in an equimolecular quantity of hydrochloric acid, the latter being employed in N/10 solution. Retrorsine was employed as hydrochloride in aqueous solution. The mice came from the same colony. In one experiment, Manske's longilobine and Adams' α- and β-longilobine were injected intravenously on the same day. In another experiment, retrorsine and β-longilobine were injected on the same day, also by vein. Five dose levels expressed by the weight of the base were used with α- and β-longilobine, and four dose levels, with Manske's retrorsine and longilobine. Groups of 5 to 10 mice were injected at each dose level. Following injection the animals were placed in individual cages for observation. All animals that died were studied pathologically as soon as possible to avoid post mortem changes. The experiment was terminated at the end of the fifth day following injection. It was our experience that if they had survived 5 days, they would recover.

The results are summarized in Table I. Those for Adams' β-longilobine from 2 tests were so close that they were combined. By inspection it will be noted that the median lethal dose ± standard error ($LD_{50} \pm$ S.E.) of longilobine matches that of β-longilobine, and the LD_{50} of α-longilobine matches that of retrorsine. When calculated by the combined slope method(10) no statistical difference could be detected among the 4 alkaloids. For example, the apparent difference in LD's between β-longilobine and retrorsine which are chemically identical is not significant at the 5% point.

TABLE I. Acute Toxicity in Mice.

Alkaloid	Dose (as base) mg/kg	No. mice died / No. used	$LD_{50} \pm$ S.E. (as base) mg/kg
Longilobine	62	2/5	76.86 ± 4.84
	70	1/5	
	80	2/5	
	90	4/5	
α-Longilobine	62	1/5	71.52 ± 4.65
	70	1/5	
	80	5/5	
	90	4/5	
	100	5/5	
β-Longilobine	62	0/5	77.20 ± 5.00
	70	2/5	
	80	9/10	
	90	6/10	
	100	10/10	
Retrorsine	56	0/5	71.67 ± 2.93
	63	3/10	
	73	5/10	
	82	7/9	

Of the mice injected, 6 died immediately—2 with longilobine, 2 with α-longilobine, and 2 with β-longilobine. No pathological examinations were made with these animals since there was not enough time for any lesion to develop. One mouse in the β-longilobine group was lost. Sixty animals that died in 7-72 hours were subjected to necropsy—7 on longilobine, 14 on α-longilobine, 24 on β-longilobine, and 15 on retrorsine.

Pathologically, the liver was unquestionably the focal organ of attack. An overwhelming majority of the livers of those mice that died from the 4 alkaloids had a mottled appearance. Many of the lobules were accentuated by central congestion with peripheral pallor. Microscopically, central necrosis with sinusoidal congestion and hemorrhage into cell cords was the predominating picture—5 with longilobine, 14 with α-longilobine, 13 with β-longilobine, and 8 with retrorsine. Infiltration of macrophages into necrotic areas was observed twice with longilobine and once with β-longilobine. In addition midzonal and periportal necrosis of the liver occurred twice with longilobine, and midzonal necrosis, once with retrorsine. One liver in the β-longilobine group showed infectious necrosis which was not attributable to the alkaloid. The livers of 10 mice were not affected by small

10. Miller, L. C., Bliss, C. I., and Braun, H. A., *J. Am. Pharm. Assn.*, 1939, v28, 644.

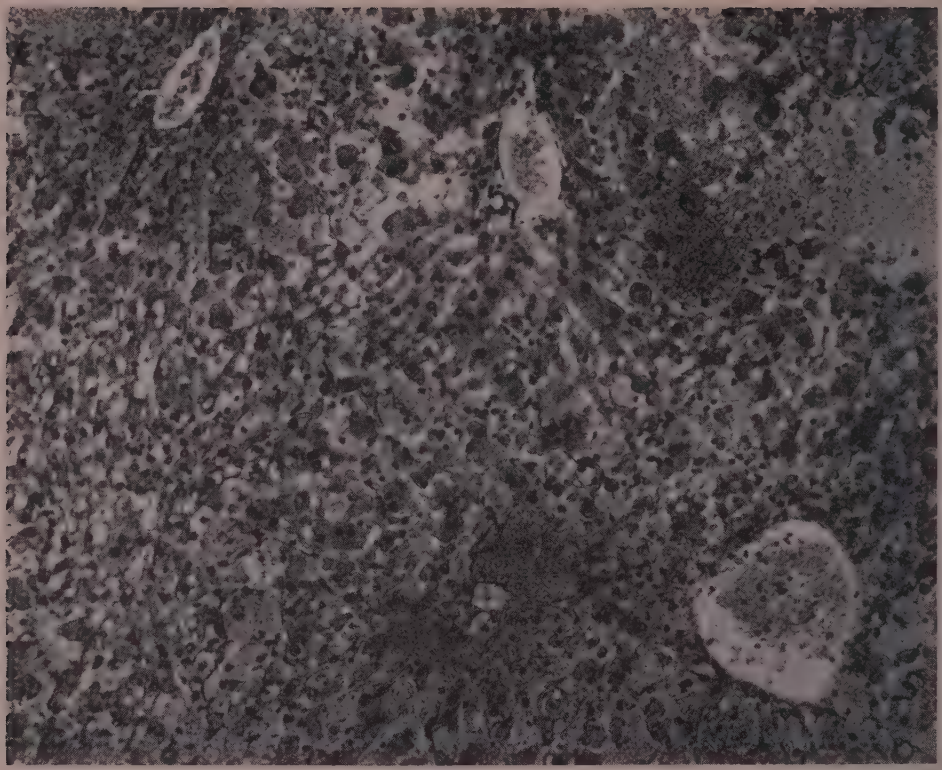

FIG. 1.

Liver damage produced by β-longilobine. This section is from the liver of an albino mouse which died 7 hr after receiving β-longilobine in a dose of 110 mg per kg by vein (Tellyesniczky's fixative, hematoxin-eosin, ×130). Note the central and midzonal necrosis and hemorrhage.

doses of β-longilobine, and those of 6 others, not by retrorsine.

The lungs were occasionally involved. Slight pulmonary oedema was present in 2 mice injected with longilobine, in 4 mice injected with α-longilobine, and in 5 mice injected with β-longilobine. In the last group, hydrothorax occurred in one animal, and broncho-pneumonia in another.

The above data indicate very clearly that all 4 alkaloids, injected intravenously to mice, have the same toxicity and the same type of hepatotoxic action. What difference exists among them elucidated by chemical means is not demonstrable toxicologically and pathologically. This is in contrast with optical isomers of sympathomimetic amines(11) and allo- and normal isomers of cardiac glycosides (12), which are easily distinguishable by pharmacological tests.

Summary. Four closely related alkaloids —longilobine, retrorsine, α- and β-longilobine —have been compared in mice by intravenous injection. There is no significant difference in their toxicity as measured by the median lethal doses. All 4 substances produce liver damage with central necrosis as the predominating lesion.

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Inhibition of Adaptive Enzyme Formation by Antimicrobial Agents. (18546)

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Despite the well-known capacity of chemotherapeutic and antibiotic agents to inhibit the growth of selected micro-organisms, the fundamental mechanisms involved in this process are poorly understood(1). The present studies indicate that the formation of certain adaptive enzymes is inhibited by several antimicrobial substances which interfere with multiplication of *Escherichia coli*. This effect provides a type of indicator reaction for studying the action of such substances on certain basic cellular processes.

Materials and methods. Suspensions of *Escherichia coli* (a freshly isolated strain furnished by the Department of Bacteriology, Army Medical Service Graduate School) were prepared from continuously aerated cultures grown for 10 hours at 37°C in brain heart infusion broth (Difco) containing 0.2% glucose. When cells adapted to lactose, maltose, arabinose, or acetate were required, they were prepared from cultures grown in a similar broth medium in which lactose, maltose, arabinose, or acetate had been substituted for glucose. Cells obtained in this manner were washed 3 times in physiological saline solution and were then aerated by a bubbling stream of air for 1 hour at room temperature prior to use. Oxygen consumption was measured in the conventional Barcroft-Warburg respirometer (32°C, 118 cycles per minute, 3 cm stroke, 3.0 ml final volume, and air as gaseous phase). Substrate concentration was uniformly 0.0017 M. Crystalline synthetic chloramphenicol and its L (+) optical isomer were obtained from Parke, Davis and Co.; terramycin hydrochloride was furnished by Chas. Pfizer and Co.; aureomycin hydrochloride was obtained in the form of the commercial intravenous preparation. The crystalline potassium penicillin G was a commercial product assaying 1435 units per mg; in the present work its concentration was expressed as mcg/ml of this commercial product.

Results. When lactose, maltose, arabinose, or acetate was added to a washed suspension of cells grown in the presence of glucose, oxygen consumption was at first equal to that of the endogenous control without substrate; however, after 40 to 120 minutes, oxygen consumption began, slowly at first but increasing exponentially until a final constant rate was attained. The results when plotted produced a curve typical of adaptive enzyme formation(2). There was no increase in number of viable organisms during the course of these experiments as determined by serial dilution plate count technic.

In contrast, when chloramphenicol (50 mcg/ml) was introduced into such a suspension prior to the addition of the substrate, the anticipated increase in oxygen consumption was not observed. Indeed, the oxygen consumption was maintained under these conditions at the level of the corresponding endogenous control (Fig. 1-B). A similar inhibi-

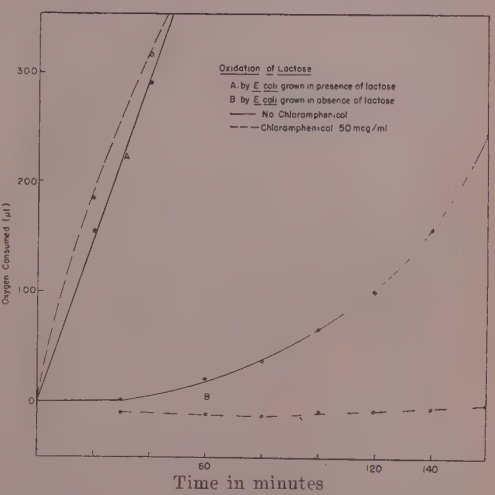

FIG. 1.
Inhibition by chloramphenicol of adaptation in *E. coli* for the oxidation of lactose and the failure of chloramphenicol to influence lactose oxidation in previously adapted organisms.

1. Pratt, R., and Dufrenoy, J., Antibiotics, Lippincott, 1949, p. 207.

2. Monod, J., *Growth,* 1947, v11, 223.

tory effect was elicited by chloramphenicol in tests on cells adapting to all 4 substrates; the data obtained from cell suspensions in the presence of lactose, illustrated in Fig. 1-B, are representative. Aureomycin (20 mcg/ml), terramycin (20 mcg/ml), and nitroacridine 3582 (50 mcg/ml) were as active as chloramphenicol in tests of this type, whereas penicillin (700 mcg/ml) failed to influence the phenomenon.

The distinction between inhibition of adaptive enzyme formation and interference with the action of the enzymes involved in the oxidation of the substrate was elucidated by the results of 2 types of experiment. In the first, cells were previously adapted by growth in a broth medium containing lactose, maltose, arabinose, or acetate instead of glucose. When 1 of these substrates was added to such a suspension of cells which had been grown in its presence, there was immediate and rapid oxygen consumption. Chloramphenicol (50 mcg/ml) did not inhibit this oxidation (Fig. 1-A).

In a second type of experiment, chloramphenicol was added at intervals to different vessels in a series during the adaptation of glucose-grown cells to lactose. Upon addition of chloramphenicol, the exponential increase in rate of oxidation was rapidly arrested. Oxygen consumption became a linear function with respect to time and the slope of the plotted curves was about proportional to the amount of adaptive enzyme present at the time chloramphenicol was added (Fig. 2). The results of this experiment confirm and supplement those of the preceding ones by demonstrating that chloramphenicol inhibits the formation of additional adaptive enzyme even though the adaptive process had already begun prior to its addition and that it does not inhibit the action of the adaptive enzyme already formed.

The L (+) optical isomer of chloramphenicol, a compound devoid of antibiotic activity (3), did not exhibit any of the effects just described.

The minimum amount of chloramphenicol

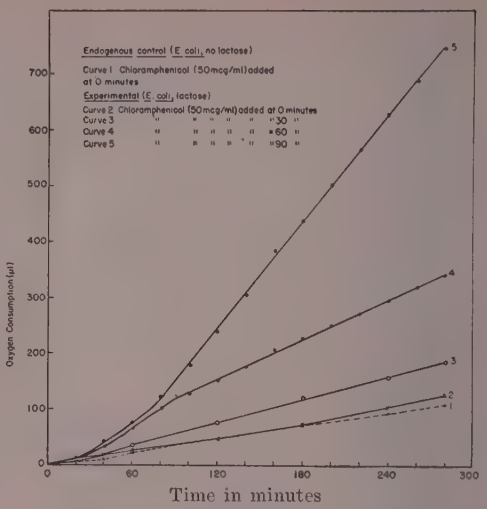

FIG. 2.

Arrest of adaptive enzyme formation in *E. coli* by chloramphenicol added at intervals during adaptation to lactose.

which would inhibit formation of adaptive enzymes in our strain of *E. coli* was approximately 5 mcg/ml. An escape from inhibition at this low drug concentration was noted after incubation of the mixture for about 2 hours in the Warburg apparatus. This escape was shown to be correlated with the reduction of the major portion of the added chloramphenicol to a biologically inactive amine, a reaction which has previously been observed in heavy suspensions of *E. coli*(4).

Washing chloramphenicol treated *E. coli* rendered them again capable of forming adaptive enzymes. *E.* coli which had been inhibited by soaking in 50 mcg/ml of chloramphenicol for 2.5 hours, in an experiment similar to that summarized in Fig. 1-B, responded in the normal manner as regards production of adaptive enzyme when washed and resuspended in lactose solution.

Discussion. The capacity of chloramphenicol to interfere with the formation of adaptive enzymes in *E. coli* appears to be related to the bacteriostatic properties of the antibiotic.* Thus, the minimal concentrations required to elicit the 2 phenomena are similar and the

3. Controulis, J., Rebstock, M. C., and Crooks, H. M., Jr., *J. Am. Chem. Soc.*, 1949, v71, 2463.

4. Smith, G. N., and Worrel, C. S., *Arch. Biochem.*, 1949, v24, 216.

L (+) optical isomer of chloramphenicol fails to produce either reaction. The minimal concentrations inhibitory to adaptive enzyme formation were not determined for terramycin, aureomycin, or nitroacridine 3582; however, the concentrations used and found effective were above the minimum bacteriostatic levels. On the other hand, adaptive enzyme formation was not inhibited by penicillin in a concentration 50 times that necessary to inhibit growth. Therefore, bacteriostatic action and inhibition of adaptive enzyme formation are frequently associated but are not inseparable properties. It may well be that the mode of action of penicillin differs from that of the other substances under investigation.

The failure of micro-organisms under the influence of chloramphenicol and other substances to form the adaptive enzymes under investigation does not account for their inability to multiply since the growth of *E. coli* with or without preformed adaptive enzymes is equally well inhibited by chloramphenicol. Adaptive enzyme formation, however, may be considered a special manifestation of a more basic cellular process—namely, protein metabolism. Experimental evidence for this point of view is furnished by Spiegelman *et al.*(5) who have shown that the adaptive formation of galactozymase in yeast is associated with the production of the specific protein component of the enzyme. In addition, Monod (6) and Reiner(7) have shown that dinitrophenol and azide, substances repeatedly demonstrated to inhibit synthetic processes, also inhibit adaptive enzyme formation. Interpretation of the inhibition of adaptive enzyme formation as either a primary or secondary interference with protein synthesis suggests a general site of action of the drugs which could readily explain their growth-inhibitory properties. Additional support for such a broad point of view is found in (a) our unpublished observations that chloramphenicol interferes with nitrogen assimilation in *E. coli,* (b) the studies of Woolley(8) suggesting that phenylalanine interferes to a limited extent with the growth-inhibitory effect of chloramphenicol, (c) the synergism and antagonism between chloramphenicol and various amino acids reported by Mentzer *et al.*(9), and (d) the recent report of Gale and Paine(10) showing that both chloramphenicol and aureomycin prevent the formation of cellular proteins in *Staphylococcus aureus.*

Summary. Chloramphenicol, aureomycin, terramycin and nitroacridine 3582 inhibit the formation of certain adaptive enzymes in *E. coli.* This effect *per se* is not responsible for the bacteriostatic activity of these substances, but it probably points toward a general interference with protein metabolism of the organism.

* Bacteriostatic concentrations were determined by incubating at 37°C for 24 hours serial dilutions of the drugs in brain heart infusion broth inoculated with the strain of *E. coli* used in this study. The minimal concentration preventing visible growth under these conditions was arbitrarily designated the bacteriostatic concentration. These levels were as follows: (1) chloramphenicol, 5 mcg/ml; (2) aureomycin, 2.5 mcg/ml; (3) terramycin, 5 mcg/ml; (4) nitroacridine 3582, 40 mcg/ml, penicillin G, 14 mcg/ml; and sodium sulfadiazine, >1000 mcg/ml.

5. Spiegelman, S., Reiner, J. M., and Morgan, I., *Arch. Biochem.,* 1947, v13, 113.

6. Monod, J., *Ann. Inst. Pasteur,* 1944, v70, 381.

7. Reiner, J. M., *J. Gen. Physiol.,* 1947, v30, 367.

8. Woolley, D. W., *J. Biol. Chem.,* 1950, v185, 293.

9. Mentzer, C., Meunier, P., and Molho-Lacroix, L., *Compt. Rend. Soc. de Biol.,* 1950, v230, 241.

10. Gale, E. F., and Paine, T. F., *Biochem. J.,* 1950, v47, 26.

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Toxicity of Colchicine, Di-isopropyl Fluorophosphate, Intocostrin, and Potassium Cyanide in Mice at 4°C. (18547)

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Toxicity studies at low ambient temperatures have been carried out predominantly in poikilotherms where the body temperature closely parallels that of the environment. These findings have been summarized by Fuhrman(1). Experiments conducted on mammals have been largely limited to drugs which impair thermoregulation. Thus it has been found that certain anesthetics are more toxic at low temperatures(2) while a calorific agent such as dinitrophenol is less toxic when the environmental temperature is lowered(1). In the present study, the effect of low ambient temperature on the toxicity of drugs which do not specifically impair thermoregulation was determined. The anticholinesterase, di-isopropyl fluorophosphate (DFP), and colchicine, a mitotic inhibitor, were employed for this purpose. Since the oxygen consumption of animals is significantly elevated at low ambient temperatures, it was thought to be of interest to investigate the toxicity of two anoxia-producing agents, potassium cyanide (KCN) and Intocostrin (Squibb) under these experimental conditions. Finally, the effect of acclimatization to the cold on the lethality of DFP was determined in one group of animals.

Method. All the mice used in the experiment were females ranging in weight from 18 to 32 g. Toxicity studies were carried out at 4°C ± 1.5°C and room temperature (23-25°C). The resting metabolism of the mice placed in the cold room was approximately 3 times basal(3), and these animals could not survive 24 hours without food. After 1 day at 4°C, the rectal temperature of the mice was depressed approximately 5°C, while in the succeeding 24 to 48 hours, the body temperature rose to within 2°C of normal. The concomitant weight loss for this period averaged only 3% (range was 0 to 10%), so that the quantity of the substances injected (on a basis of mg/kg) was essentially the same in both experimental and control animals. The natural mortality of 140 untreated controls was 22% for the first 24 hours following exposure to the cold, and thereafter it averaged less than 5% per day. This was compensated for in the statistical evaluation of the LD_{50}s. The mice were kept in toxicity cages supplied with food (Purina Fox Chow) and water. Each section of the cage was 7″ x 4″ x 3″ and contained 2 animals. Ten animals were injected subcutaneously at each dose and 5 dosages were employed in each determination. Colchicine, KCN, and Intocostrin were administered in saline in a volume of 0.2 ml/20 g while DFP was injected in propylene glycol in a volume of .05 ml/20 g. Control injections of 0.5 ml of saline or 0.1 ml of propylene glycol had no influence on the natural mortality of the animals. The mice were injected 24 hours after they were placed in the cold room and were maintained at 4°C until the fatalities were counted. This was done 72 hours after colchicine was administered, 48 hours after DFP, and 2 hours after KCN and Intocostrin were injected. Controls at room temperature were simultaneously injected with the same solutions. Acclimatized mice were treated with DFP 28 days after they were placed in the cold room. These latter mice were kept in ordinary cages with 25 animals per cage for 3 weeks (reducing their natural mortality for this period to 1% per day) and were placed in individual cages as described above 1 week before injection. The LD_{50}s were calculated by the method of Bliss(4) with ap-

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1. Fuhrman, F. A., *Physiol. Rev.*, 1946, v26, 247.
2. Hermann, J. B., *J. Pharm. and Exp. Therap.*, 1941, v72, 130.
3. Brody, S., Bioenergetics and Growth, Reinhold Publishing Corp., New York, p. 284, 1945.
4. Bliss, C. I., *Quart. J. Pharm. and Pharmacol.*, 1938, v11, 92.

TABLE I. Toxicity of Various Compounds Injected Subcutaneously in Mice.

Compound	23-25°C LD_{50} (mg/kg) and std. error	4°C LD_{50} (mg/kg) and std. error	Approx. incr. in toxicity at 4°C, %
a. Colchicine	3.10 ± .20	2.32 ± .20	33
b. DFP	4.67 ± .28	3.22 ± .31	45
c. Intocostrin	.67 ± .05	.38 ± .03	76
d. KCN	6.02 ± .33	2.86 ± .16	110
e. DFP in acclimatized mice	4.43 ± .36	4.76 ± .50	None

propriate compensation made for the natural mortality of the mice in the post-injection period. The transformed dosage-mortality curves were found to be statistically parallel, so that the toxic mode of action of the drugs was assumed to be similar in both groups. Therefore the dosages calculated for the LD_{50}s in the two sets of experiments are directly comparable.

Results. The results of all experiments are shown in Table I. All drugs were significantly more toxic ($P<.01$) in mice that had been injected 24 hours after exposure to the cold than in controls (Table I-a, b, c, and d). In order to obtain the LD_{50}s at 4°C, as compared to room temperature, the dosages had to be decreased 25 to 52% depending upon the drug employed. Thus the toxicity of KCN was more than doubled and that of Intocostrin was increased 76%. The potency of the two drugs which did not interfere with oxygen utilization, colchicine and DFP, were increased 33 and 45% respectively. However, the acclimatized mice were no more susceptible to DFP than were the controls that were treated at room temperature (Table I-e).

Discussion. Fuhner(5) and Sanno(6) found that the toxicity of colchicine in frogs was increased 400 to 500 times when the temperature was raised 12°C, *i.e.*, from 20 to 32°C. Also, Hausmann(7) reported that hibernating bats were immune to 30 times the dose of colchicine lethal to non-hibernating bats. Interestingly enough, the lethal dose of colchicine in frogs at 30°C is approximately the same as for mice, about 3 mg/kg(8,9). Earlier work led Jacobj(10) to suggest that the toxicity of the compound was a function of body temperature, the toxic principle being the oxidation product, oxydicolchicine. However, in poikilotherms, body temperature can not be dissociated from the metabolic rate. Mammals, on the other hand, maintain a relatively constant body temperature while their metabolic rates may fluctuate considerably. In the present experiment, colchicine was injected into slightly hypothermic mice whose oxygen consumption was at least twice basal. Under these conditions, the toxicity of the drug was only 0.33 times higher than the controls, as compared to the 400-fold increase in the frogs whose oxygen consumption was doubled (assuming $Q_{10} = 2$) when their body temperature was increased 10°C. This lends further support to the hypothesis that the toxicity (oxidation ?) of colchicine is related to the body temperature rather than to the general metabolic level of the animal.

The increased toxicity of KCN (110%) and Intocostrin (76%) is not surprising in view of the augmented metabolic rate of the mice at 4°C. The lethality of Intocostrin is due to its curare-like action on the muscles of respiration, while KCN inhibits cytochrome oxidase and thus prevents the utilization of oxygen by the tissues. The increased potency of KCN approximated to a large extent the increment in the metabolic rate of the animals in the cold. If the increased oxygen consumption is accompanied by the utilization of

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6. Sanno, J., *Arch. f. exper. Path. u. Pharmakol.*, 1911, v65, 325.

7. Hausmann, W., *Pflugers Arch.*, 1906, v113, 317.

8. Fuhner, H., *Arch. f. exp. Path. u. Pharmakol.*, 1932, v166, 437.

9. Fuhner, H., and Breipohl, W., *Arch. f. exp. Path. u. Pharmakol.*, 1933, v173, 146.

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a greater percentage of the available cytochrome oxidase, it seems logical that lethality in the cold would occur when a proportionately smaller fraction of the total enzyme was inhibited.

Mice that were acclimatized to the cold were no more susceptible to DFP intoxication than were the controls treated at room temperature, while the same drug was 45 per cent more potent in mice that had been exposed to the cold for only 24 hours before injection. The anatomical and functional changes that occur in acclimatization to the cold are not entirely understood. However, several workers have reported significant increases in the kidney and liver weight as a function of long exposure to low ambient temperatures(11,12). These findings, confirmed by recent work done in this laboratory, indicate that the detoxification potential of these tissues in cold-acclimatized animals may be greater than in unacclimatized or normal animals. This phase of toxicology obviously requires more study. However, the generalized decreased resistance of the animals to drugs after short exposure to the cold indicates that these mice were essentially weaker and somewhat hypothermic animals under stress. This is borne out by the relatively high natural mortality evident during the first 24 hours at 4°C.

Summary. The toxicity of colchicine, DFP, Intocostrin and KCN was increased in mice that were exposed to 4°C for 24 hours and maintained in the cold after injection. Animals that were acclimatized to the cold were no more susceptible to DFP than were controls treated at room temperature. The possible factors implicated in these findings are discussed.

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Influence of Histamine upon Fungistatic Action of Antihistaminics. (18548)

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The work of Carson and Campbell(1), which reported the fungistatic action of 3 antihistaminics, prompted an investigation concerning which the following is a preliminary report. In addition to testing fungistasis by several antihistaminics, we were also interested in determining whether the compounds are fungicidal as well. Lastly, it was considered of fundamental biochemical interest to determine whether the antifungal action of antihistaminics is based on interference with utilization of histamine (or a compound or compounds of similar structure) by the fungus.

The compounds studied were as follows: Benadryl, (Diphenhydramine HCl) Parke-Davis; Thephorin, (Phenindamine Tartrate) Hoffmann-La Roche; Thenylene, (Methapyriline HCl) Abbott; Neo-Antergan, (Pyranisamine Maleate) Merck; Pyribenzamine, (Tripelenamine HCl) Ciba, and Diatrine, (Metaphenilene HCl) Wm. R. Warner.* The test culture employed was Trichophyton mentagrophytes (Emmons No. 640).

1. Carson, L. E., and Campbell, C. C., *Science*, 1950, v3, 689.

* The authors gratefully acknowledge supplies of antihistaminics by Dr. Graham Chen, Parke-Davis & Co.; Dr. R. K. Richards, Abbott Laboratories; Dr. H. Molitor, Merck and Co.; Dr. F. F. Yonkman, Ciba Pharmaceutical Co., and Dr. J. A. Aeschlimann, Hoffmann-La Roche.

TABLE I. Activity of Antihistaminics Against *T. mentagrophytes.*

Compound	Highest active dilution (1:)	Highest active dilution (1:) With histamine mg/.5 ml vol. .05	.1	.25	.5	.75	1	2.5	5	With histidine mg/.5 ml vol. 2.5
Thenylene	6400	6400	6400	6400	3200	400	800	400	400	3200
Benadryl	12800	25600	12600	12800	3200	800	800	400	800	12800
Thephorin	51200	51200	51200	51200	3200	400	400	800	800	51200
Neo-Antergan	6400	3200	6400	3200	800	400	400	400	200	6400
Diatrine	25600	25600	25600	25600	800	800	800	400	400	25600
Pyribenzamine	6400	12800	6400	12800	6400	800	400	400	800	6400
Desenex	25600	51200	25600	25600		25600	25600		51200	25600

Methods. The method of testing for fungicidal action was that of Oster and Golden(2). The fungistatic test technic was a serial dilution method using Sabouraud's maltose broth medium in a series of 15 test tubes (75 x 12 mm with aluminum caps to facilitate pipetting) for each test preparation. In each tube except the first was placed 0.5 ml of the base medium. To tubes 1 and 2 were added 0.5 ml of a 1.0% solution of the test material dissolved in the medium. Beginning with tube No. 2, the contents were well mixed and 0.5 ml transferred to the next tube. The same procedure was continued through tube 14 from which 0.5 ml was discarded. Tubes 1 and 15 were controls. With a No. 2 cork borer, inoculum disks 2 mm in diameter were cut from an agar plate culture 12-15 days of age, and were introduced into each of the tubes. Incubation was at 25°C and at the end of 2-3 days initial estimates of growth were made.

Results and discussion. The results obtained indicate that Benadryl, Thephorin, Diatrine, Thenylene, and Pyribenzamine do not exert any fungicidal activity in concentrations of 1.0%, 2.5%, 5.0% and 10.0%. Diatrine and Thephorin, being water insoluble above 2.5%, were tested as suspensions above this concentration and the results, therefore, may not be a true indication of their fungicidal capacity in other solvents making possible higher concentrations. The fungistatic activity of Pyribenzamine appears to be of the order reported by Carson and Campbell. The fungistatic action of the other antihistaminics tested, as is evident in the left column of the table, may be listed in the following descending order of effectiveness: Thephorin, Diatrine, and Benadryl; Pyribenzamine, Thenylene, and Neo-Antergan. It is interesting to note that the action is completely prevented by certain amounts of histamine. By the addition of varying amounts ranging from 0.05 mg to 5.0 mg to each 0.5 ml volume, it was found that in most cases the prevention of fungal growth by the antihistaminic was moderately overcome in the presence of at least 0.25 mg, and completely overcome with 0.75 mg (/0.5 ml volume) of histamine. Concentrations below 0.25 mg were ineffective (See table). On the other hand, histidine had no such effect on our fungal strain with as much as 2.5 mg/0.5 ml, although Robbins and Ma (3) reported in 1945 that histidine augmented the growth of *Trichophyton mentagrophytes* in a casein hydrolysate medium. Whether or not the need for histidine noted by Robbins and Ma is to supply a source of histamine, our observation on histidine seems to indicate that in the presence of the antihistaminics the organism is unable to decarboxylate histidine in sufficient quantity to produce the effect. This aspect of the problem is being studied. An obvious hypothesis to explain the action of histamine herein described is that *T. mentagrophytes* requires histamine (or a closely similar compound) for growth and that the cellular chemical topography concerned is "blanketed" or blocked by the antihistaminics; the reversal is apparently competitive, for the degree depends on histamine concentration within certain limits. Since none of the antihistaminics tested

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3. Robbins, W. J., and Ma, R., *Am. J. Botany,* 1945, v32, 509.

are fungicidal under conditions of the study, the process affected is evidently concerned with the growth alone, and not with the survival of the resting organism. That this property of histamine is specific toward the antihistaminics is evident from the fact that the fungistatic action of 10% undecylenic acid (as Desenex) is not affected. Additional work with other strains as well as on the underlying mechanism of action is now being pursued and will be reported at a future date. From the practical standpoint not only may the allergic component in host tissue response to dermatophytoses be controlled by the use of antihistaminics (in conjunction with conventional fungicidal preparations), but also some fungistatic action by the antihistaminics themselves might be expected. Finally, our results throw light on the mechanism of fungistatic action of the antihistaminics; thereby histamine (or chemically related substances), liberated by infected host tissue, could not be utilized for growth by the organism.

Summary. Several antihistaminics are fungistatic, preventing the growth of *T. mentagrophytes*. The effect is prevented by suitable amounts of histamine, but not by histidine.

While this report was in preparation, Reiss *et al.* published similar results with Trimeton Maleate (Exp. Med. and Surg., 1950, v8, 330) with reference to histamine.

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Protection by Flavonoids against Histamine Shock. (18549)

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Wilson, Mortarotti and DeEds(1) reported that pre-treatment with rutin resulted in slight, though definite, protection of guinea pigs given LD_{50} doses of histamine. There was a previous report by Hiramatsu(2) that hesperidin afforded protection of guinea pigs against anaphylactic shock, and Parrot and Richet(3) had found that the increased sensitivity of scorbutic guinea pigs to histamine was returned to normal after administration of D-catechin isomers. Since publication of the paper by Wilson *et al.*, there have been a number of other reports on the subject. Clark (4) stated that rutin and quercetin afforded protection against histamine; others(5-11) were unable to confirm this. Three papers (5,9,10) described a protection against anaphylactic shock, while three others(4,7,8) reported no protection. In addition to the above direct evidence, there are reports of beneficial effect of flavonoids in peptone shock (12), in the Shwartzman phenomenon(13), in an anaphylactoid reaction(14), in drug sensitization(15), and in allergic conditions in man(16,17). Shanno(18) stated that ru-

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2. Hiramatsu, N., *Japanese J. Dermat. Urol.*, 1941, v49, 304.

3. Parrot, J.-L., and Richet, G., *Compt. rend. soc. biol.*, 1945, v139, 1072.

4. Clark, W. G., *Am. J. Physiol.*, 1949, v159, 564.

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10. Moss, J. N., Beiler, J. M., and Martin, G. J., *Science*, 1950, v112, 16.

11. Semenza, F., *Acta Vitaminologica*, 1949, v3, 257; Abstracted in *Excerpta Med.*, 1950, v3, Sec. II, 1112, Item 4574.

tin was of no benefit in 3 cases of drug sensitivity. Peña(19) found that histamine wheals in man were smaller in size and disappeared more rapidly when rutin was administered.

The conflicting findings mentioned above indicate that the experiment is not as simple as it appears at first glance. In this paper we present data obtained in an attempt to repeat our earlier work with rutin, and in addition we have studied 3 other flavonoids for possible protection of guinea pigs injected with histamine.

Materials and method. Guinea pigs were purchased from local animal supply firms, and represented a heterozygous stock. After becoming acclimated to the conditions of the laboratory, the animals were placed on a scorbutic diet supplemented with ascorbic acid, as previously described(1), for a week or two, in order to deplete them of flavonoid materials. Body weights at time of test varied from 250 to 540 g, with 75% of the animals weighing from 310 to 465 g. Body weight, within the above limits, and sex did not appear to influence either the reaction to a given dosage of histamine, or the effect of flavonoids on histamine shock. What did affect susceptibility to histamine was the particular batch of animals. It was necessary to order 4 or more dozen animals at a time in order to have a sufficient number of experimental pigs available after standardizing the group against histamine. The dosage of histamine was critical and varied from batch to batch of animals. The variation was not seasonal. Histamine was given as the hydrochloride, with the approximate LD_{50} dose ranging from 0.20 to 0.25 mg of histamine base/kg. Injection was into the jugular vein after the vein had been exposed following procaine anesthesia. A 5% change in dosage was sufficient to change an LD_{50} to an LD_0 or an LD_{100}. To demonstrate a protective effect of flavonoids on histamine shock, an LD_0 was useless, of course, and the action of flavonoids is so slight that a clear demonstration of protection was not obtained if a dosage of histamine near the LD_{100} level were used. A brief discussion of this point has been published(20). All of the flavonoids* were given intraperitoneally, 40 mg/animal, 30 minutes before histamine. At this high level, which was chosen deliberately so as to get maximum intraperitoneal absorption, there was occasional precipitation within the peritoneal cavity, especially in the case of quercetin. Eight per cent solutions of rutin and quercitrin were made in 33% propylene glycol with the aid of heat and a trace of alkali ($NaHCO_3$). A 20% solution of quercetin in straight propylene glycol permitted injection of a small enough volume (0.20 ml) to cause minimal peritoneal irritation. The hesperidin methyl chalcone was dissolved in saline to give an 8% solution. All control animals received intraperitoneal injections of the appropriate solvents.

Results. The results are presented in Table I. The data on rutin-treated animals is divided into 2 parts. The earlier data were obtained from small groups of inadequately standardized animals, and indicate no protection against histamine. Later tests with more carefully standardized animals show that rutin gives some, though not marked, protection.

The other flavonoids were given to somewhat fewer animals, but the protection is more apparent. The animals pre-treated with

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15. Hüllstrung, H., and Hack, K., *Z. Immunitäts.,* 1941, v100, 393.

16. Isaacs, R., *Proc. Central Soc. Clin. Res.,* 1944, v17, 66.

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18. Shanno, R. L., *Am. J. Med. Sci.,* 1946, v211, 539.

19. Peña Regidor, P. de la, Cavero, F., and Segarra, F., *Rev. Clin. Española,* 1950, v37, 118.

* The rutin was furnished by the Eastern Regional Research Laboratory, of this Bureau; the methyl hesperidin chalcone was kindly supplied by the California Fruit Growers' Exchange. The quercetin and quercitrin were isolated from lemon flavine by a recently-reported procedure(21).

20. Wilson, R. H., and DeEds, F., *Science,* 1948, v107, 369.

21. Booth, A. N., and DeEds, F., in press.

TABLE I. Survival of Histamine-Treated Guinea Pigs After Pre-Treatment with Flavonoid Compounds.

	Controls		Experimental	
	No. surviving / No. injected	% surviving	No. surviving / No. injected	% surviving
Rutin (early tests)	9/15	60	10/15	67
" (later tests)	8/15	53	16/20	80
Quercitrin	4/7	57	5/5	100
Quercetin	3/10	30	8/10	80
Methyl hesperidin chalcone	2/7	29	6/11	55

quercetin were especially noteworthy, in that survivors had no, or exceptionally slight, symptoms of histamine shock.

Discussion. There are two points at which modification of technic can change positive to negative results: the time between flavonoid administration and histamine injection (1), and histamine dosage, which may vary with each batch of animals. A critical consideration of the various papers concerned with the effect of flavonoids on histamine shock shows that the main variable was dosage of histamine. In most cases where negative results were reported, the amount of histamine was great enough to kill all, or nearly all, of the control animals. Since the protection by rutin is slight, it is much less apt to be apparent at high histamine levels. With a sufficiently large group of animals carefully standardized against histamine, we found protection. When less care was taken to standardize the animals, and when amounts of histamine approaching an LD_{100} were used, we were unable to show any effect from the flavonoids. Incidentally, it has been our experience that the slope of the histamine curve in any one group of animals is very steep, so that small changes in dosage have a profound effect on mortality.

The three other flavonoids likewise afforded protection, with quercetin appearing to be the best of the substances tested. The exact degree of protection cannot be judged by these smaller groups. Clark(4) reported that he found quercetin sulfonate more efficient against histamine than was rutin.

It should be emphasized that the flavonoids are not antihistaminics. Not only is their protective action very slight, but there is evidence(1,8,9,19) that there is no direct antagonism between rutin and histamine. The reaction is indirect and is important only in suggesting the mode of action of the flavonoids.

Summary. A slight protection of guinea pigs treated with rutin and subjected to an LD_{50} dose of histamine has been confirmed. The failure of certain other investigators to substantiate this finding may have been due to excessive histamine dosage or the use of inadequately standardized animals. Quercitrin, quercetin and methyl hesperidin chalcone also were found to afford protection against histamine. The protection is slight and is of theoretical rather than clinical interest.

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Effect of Hydrolytic Products of Procaine on Procaine Toxicity and Metabolism in Mice.* (18550)

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During previously reported studies on the effect of cholinesterase inhibitors on the toxicity of procaine in mice(1) a preliminary test of the effect of diethylaminoethanol (DEAE) in this regard was made, since Velázquez *et al.*(2) had observed that this hydrolytic product of procaine exerts some cholinesterase inhibiting action. This test showed that pretreatment with DEAE significantly increased the toxicity of procaine in mice. Considering the possibility that this could be due to a competition between DEAE and procaine for procainesterase(3,4) we have extended the observations with DEAE and have also included para-aminobenzoic acid (PABA), the other hydrolytic product of procaine, and dimethylaminoethanol (DMAE), a close analogue of DEAE. Tests have also been carried out to determine whether the rate of disappearance of procaine *in vivo* is altered by pretreatment of mice with these three agents.

Materials and methods. In order to be able to select doses of PABA, DEAE, and DMAE which were approximate toxic equivalents in mice, and to choose the time intervals between the administration of premedicants and procaine which would permit the development of the maximum action of the former, preliminary LD_{50} determinations were made on each of these agents and the death times were observed. Healthy Carworth white mice of both sexes weighing 20-30 g were used. The PABA solution, neutralized with sodium hydroxide, was prepared in a 25% concentration for the determination of its LD_{50}, and in a 7% concentration for the premedication in the procaine toxicity tests. Both DEAE and DMAE were 5% in concentration and neutralized with hydrochloric acid. In the toxicity study 1% procaine hydrochloride was used, and in the *in vivo* hydrolysis study a 0.5% solution was employed. All drugs were made up in 0.9% saline solution. PABA, DEAE, and DMAE were administered subcutaneously, while the procaine was injected into a tail vein in all cases. The LD_{50} and its 5% fiducial limits was calculated by means of probit analysis (5).

In the study of the influence of the various subcutaneous premedications on the rate of procaine hydrolysis *in vivo*, all mice were fasted for 7 hours before receiving the procaine injection. At the end of the fasting period, 30 mg/kg of procaine was injected intravenously. At various time intervals after the procaine injection the mice were dropped into a Waring Blendor and homogenized for 60 seconds in 3 ml of 4% trichloro-acetic acid for each gram of body weight. The trichloro-acetic acid precipitated the protein and stopped the procaine hydrolysis instantly. The homogenate was then filtered. Three ml of the filtrate were used for the measurement of procaine by a spectrophotometric method(6). The procaine content of the mouse body at zero time was obtained by dropping the mouse into the homogenizer immediately after the procaine. This maneuver took only a few seconds and gave the tissues little chance to destroy the procaine.

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5. Finney, D. J., *Probit Analysis*, 1st ed., Cambridge University Press, 1947.

6. Ting, K. S., Coon, J. M., Conway, A. C., *J. Lab. and Clin. Med.*, 1949, v34, 822.

TABLE I. Subcutaneous Toxicity of PABA, DEAE, and DMAE in Mice.

	No. of mice	LD_{50} (g/kg)	5% fiducial limits of LD_{50} (g/kg)	Time of most deaths
25% PABA	60	4.39	4.20-4.59	7-24 hr
5% DEAE	65	1.61	1.08-2.07	15-60 min.
5% DMAE	110	2.08	2.04-2.12	15-60 min.

Results. Following the injection of lethal doses of PABA the mice became inactive, weak, and drowsy in 30 to 60 minutes and prostrate in several hours. Most of the deaths occurred in 7 to 24 hours but a few died as long as 48 hours after the injection. After the administration of DEAE the animals succumbed in 15 to 60 minutes. There were no delayed deaths. Following the injection of DMAE most of the mice died in 15 to 60 minutes, but some died after having remained prostrate for two to three days. The deaths occurring within an hour were generally preceded by convulsions. Mice dying after one day manifested no convulsions, but severe prostration and gasping were seen for many hours before death. The toxicities of PABA, DEAE, and DMAE, and the range of death times in mice following injections of these substances are illustrated in Table I.

With these data on hand, the LD_{50} of intravenous procaine was determined on mice premedicated with PABA, DEAE, or DMAE. The dose of each premedicant used, the interval of time allowed between its administration and the injection of procaine, and the procaine toxicity results obtained are indicated in Table II. In each case the dose of premedicant was approximately one third to one fourth the LD_{50} of the premedicant and the time interval allowed before the injection of the procaine was that which had been observed necessary to elapse before deaths began to occur following administration of the LD_{50} of the premedicant. An additional test of the toxicity of procaine was carried out on a group of mice which had been pretreated with PABA only 30 minutes before receiving the procaine. The doses of the premedicants employed produced no noticeable toxic effects on the animals. The tabulated results show that both DEAE and DMAE diminished the LD_{50} of intravenous procaine by approximately 35%, while PABA, given either 30 minutes or 7 hours before the procaine, had no influence upon the toxicity of the latter drug.

In the study of procaine hydrolysis *in vivo,* 60 mice were used in the control group and in each of the groups receiving PABA, DEAE, or DMAE. The curves in Fig. 1 relate time after injection with the percentage of the total amount of injected procaine recovered in the whole mouse homogenate. The differences between the results obtained with the control group and with the premedicated groups were all statistically non-significant.

Discussion. It could not be demonstrated that pre-treatment by DEAE, PABA, or DMAE had any effect on the rate of disappearance of intravenously administered procaine from the whole mouse body. Thus it may be presumed that if procainesterase plays an important role in the *in vivo* hydrolysis of procaine, this role is not interfered with in any way by the hydrolytic products of procaine or by DMAE. This finding indicates that the increase in the sensitivity of mice

TABLE II. Toxicity of Intravenous Procaine in Mice After Subcutaneous Premedication by PABA, DEAE, and DMAE.

Subcut. premedicant	No. of mice	Time interval between premedication and procaine	LD_{50} of procaine (mg/kg)	5% fiducial limits of LD_{50} (mg/kg)	Significance of diff. from control LD_{50}
Control	50	—	50.7	47.7-54.2	
7% PABA, 1 g/kg	46	7 hr	50.7	42.0-63.4	Non-sig.
7% PABA, 1 g/kg	50	30 min.	53.1	49.4-56.2	Non-sig.
5% DEAE, .5 g/kg	85	15 min.	32.5	30.9-34.0	Highly sig.
5% DMAE, .5 g/kg	40	15 min.	34.2	32.5-36.5	Highly sig.

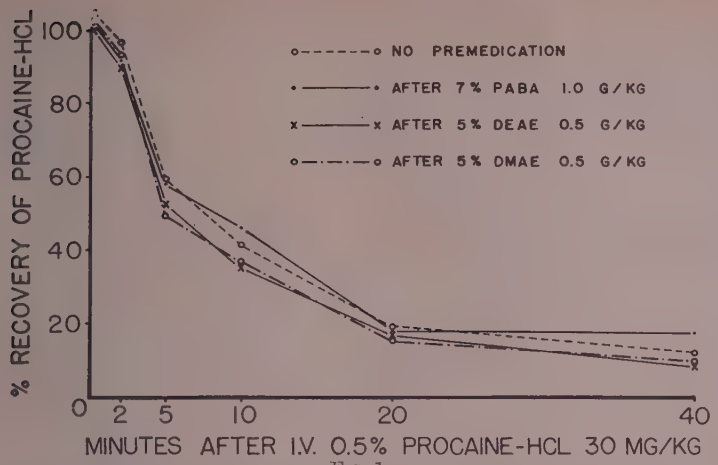

FIG. 1.

Disappearance of intravenously administered procaine in control mice and in mice pretreated subcutaneously with PABA, DEAE, and DMAE. (Each point represents the average of 10 determinations on 10 mice).

to the lethal action of intravenous procaine following pretreatment with apparently nontoxic doses of DEAE and DMAE may be the result of the additive actions of these materials and procaine. These additive actions seem most likely to be on the cardiovascular system. Following rapid intravenous injection procaine causes death primarily through its effect on the heart(7-9). Therefore, premedication by relatively small doses of drugs having an effect on the heart or vascular system could conceivably lower the threshold for the toxic dose of intravenous procaine. Observations by other workers indicate that DEAE and DMAE possess actions which might be of some value in interpreting the influence of these agents on the toxicity of intravenously administered procaine. DEAE has been shown to cause inhibition of the sympathetic vasoconstrictor reflex in man (10), dilatation of peripheral vessels in mammals(11), and hypotension(12,13). Both procaine and DEAE inhibit cholinesterase but PABA does not(2). DMAE may slightly lower the blood pressure in the dog(14), and in toxic doses this agent produces auriculoventricular dissociation(15), pulmonary edema and death(16).

PABA has little effect on the cardiovascular system, and would not be expected to exert any influence on the intravenous procaine toxicity in mice. The subcutaneous LD_{50} of 25% PABA (neutralized with sodium hydroxide) in mice was found to be 4.39 g/kg which is very close to the intravenous LD_{50} demonstrated by Scott and Robbins(17) and by Richards(18) for rats injected intraperitoneally. It is significant that PABA, DEAE, and DMAE are all less toxic than procaine which has a subcutaneous

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LD_{50} of 968 mg/kg in mice(19).

The present results are of some interest in the light of the work of Richards and Kueter(20) who observed that the incidence of procaine convulsions in guinea pigs was reduced by the prior administration of either DEAE or PABA, or of both of these substances simultaneously. They presented evidence favoring competitive inhibition as the mechanism of this phenomenon. They observed however that PABA and DEAE had no inhibitory effect on the local anesthetic action of procaine, nor on procaine-induced convulsions in mice. Our results further fail to show in mice any evidence of competition between procaine and these substances for procainesterase, or for any other receptor substance or structure which may be involved in the gross lethal action of intravenously administered procaine.

Summary. The subcutaneous LD_{50}s of 25% PABA, 5% DEAE, and 5% DMAE in mice were found to be 4.39, 1.61, and 2.08 g/kg respectively. Subcutaneous premedication by 1 g/kg of 7% PABA did not alter the LD_{50} of intravenous procaine in mice, while subcutaneous premedication by 0.5 g/kg of either 5% DEAE or 5% DMAE lowered the LD_{50} about 35%. Neither PABA, DEAE, nor DMAE changed the rate of disappearance of procaine from the whole body of the mouse. It is concluded that the influence observed to be exerted by DEAE and DMAE on the toxicity of intravenous procaine is probably due to their additive toxic effects on the cardio-vascular system, and not to the inhibition of procainesterase *in vivo*.

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Phosphocreatine and Adenosine Triphosphate Content of Rat Tissues after Adrenalectomy and Cortisone Treatment (18551)

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In studies on the metabolic effects of adrenalectomy and hormone treatment, the question frequently arises as to whether adrenalectomized animals lack an adequate supply of readily available energy. One index of their energy supply is the adenosine triphosphate (ATP) and phosphocreatine content of the tissue. Data on the ATP and phosphocreatine content of rat tissue from adrenalectomized animals are somewhat inconsistent(1). Part of this confusion is due to lack of correction for salt balance and part is probably due to earlier inadequate methods for measuring ATP. It was therefore of interest to determine the ATP and phosphocreatine content of tissues of animals under various conditions of treatment. Using rats maintained on saline we have found no significant alteration in the phosphocreatine or ATP content of muscle after adrenalectomy or after treatment with cortisone. All data presented are on animals starved for a period of 24 hours before analysis, since this condition might be likely to result in altered phosphocreatine. No difference was found between well-fed or starved animals.

Methods. A. *Preparation of animals.* The rats, weighing approximately 150 g were adrenalectomized bilaterally in the laboratory of Dr. H. C. Stoerk to whom we are indebted for this material. After adrenalectomy, the animals were supplied an adequate diet and

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TABLE I. Phosphocreatine Content of Rat Muscle.

Treatment		Phospho-creatine, mg %	Inorganic phosphorus, mg %	Total phosphorus, mg %
Normal, untreated		257	45	—
		248	24	—
		305	45	—
		253	31	131
		244	30	134
		270	29	116
		284	34	135
	Avg	266 ± 20*	34 ± 7	129 ± 8
Adrenalectomized, 10 days		248	33	118
10		259	25	109
10		301	25	125
10		303	23	—
3		271	13	—
3		326	24	—
7		310	35	—
26		297	35	—
	Avg	289 ± 25	27 ± 7	117 ± 7
Normal, treated with cortisone, 10 days		261	28	114
		326	28	125
		279	34	125
	Avg	288 ± 27	30 ± 5	121 ± 5
Adrenalectomized, treated with cortisone, 10 days		303	32	135
		244	24	104
		260	27	117
	Avg	269 ± 24	28 ± 3	119 ± 11

* Stand. dev. of the mean.

0.9% NaCl in place of drinking water. Treatment with cortisone consisted of the intramuscular injection of 1 mg Cortone* per day supplied as a single daily dose. Animals were starved by removing their food for 24 hours prior to analysis.

B. *Analytic procedures.* The animals were injected intraperitoneally with 5 mg/100 g of pentobarbital. At the end of 10 minutes, when they were in surgical anesthesia, the skin was freed around the left gastrocnemius muscle. The muscle was dissected from the underlying fascia and the skin replaced over the muscle. At the end of 15 minutes the muscle was once more exposed, cut at its origin and at its insertion, and quickly dropped into liquid air; the removal of the muscle was completely within 10 seconds. This system of preparation of tissue for analysis gives results comparable to those obtained by freezing the whole animal(2).

The tissue was fragmented without allowing it to thaw and transferred to tared centrifuge tubes containing 5 cc of cold trichloracetic acid. The supernatant fluids were collected by centrifugation and the muscle residues extracted once with 2 cc of 10% trichloracetic acid and then with 2 cc of 5% trichloracetic. The combined supernatants were made to a volume of 10 cc. Samples were immediately withdrawn for determination of true inorganic phosphorus and total inorganic phosphorus according to the method described in Umbreit *et al.*(3). Determinations of the total phosphorus were also carried out on some samples. The remainder of the trichloracetic acid extract was neutralized and precipitated with

* Cortone (Merck), consisted of cortisone acetate formulated in suspending agents and containing 1.5% benzyl alcohol as a preservative.

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3. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., 1949, Manometric Technics and Tissue Metabolism, Minneapolis.

barium acetate in the presence of 3 volumes of alcohol. The barium alcohol insoluble precipitate was collected, freed of barium and assayed for adenylic acid, adenosine diphosphate and adenosine triphosphate according to the method described by Albaum and Lipshitz(4). The method briefly consists in enzymatically converting adenosine diphosphate and adenosine triphosphate into adenylic acid and determining the latter as inosinic acid in the spectrophotometer.

Results. Data on the muscle phosphocreatine content of starved animals under various treatments are given in Table I. It is evident that adrenalectomy or cortisone treatment did not significantly alter the phosphocreatine content of the muscle; nor was there any effect of starvation. Well fed animals had the following phosphocreatine content in the muscle: Normal, 258 mg %, adrenalectomized, 281 mg %. It seems apparent that the adrenalectomized animal had as much phosphocreatine content in the muscle as the normal or cortisone treated animal and that, therefore, lack of phosphocreatine was not the cause of muscle fatigue or of increased sensitivity to stress. While the amounts of phosphocreatine found are very low (approximately 20 mg %) we were also able to find no change in the phosphocreatine content of kidney or liver upon adrenalectomy, with or without starvation.

TABLE II. Adenosine Triphosphate Content of Rat Muscle.

Treatment	ATP, mg %	Avg
Normal, no treatment	262 224 242	242 ± 15
Adrenalectomized	265 235 263	254 ± 14
Normal, treated with cortisone, 10 days	200 223 215	213 ± 10
Adrenalectomized, treated with cortisone, 10 days	262 236 202	233 ± 24

Since phosphocreatine is not altered one would expect no change in the ATP and since phosphocreatine is relatively high, one would further expect to find little adenosine-diphosphate or adenylic acid in the muscle. None of the muscle samples contained adenosine-diphosphate or adenylic acid. Their ATP content is given in Table II from which it is evident that there is no significant change in ATP content.

Conclusion. There was no significant effect of adrenalectomy or cortisone treatment upon the phosphocreatine or ATP content of rat muscle.

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Diasone and Promin as Therapeutic Agents in Experimental Toxoplasmosis.* (18552)

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Since this paper is concerned with the problem of therapy in toxoplasmosis, other pertinent information with respect to this protozoan disease should be sought elsewhere (1-4). 2(para amino benzene sulfonamide) pyridine (Sulfapyridine) and 2(p-acetyl-

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† Acknowledgement is made of the technical assistance of Bernard Schnitzer and Eugene Winograd.

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aminobenzene sulfonamide) thiazole (Sulfathiazole), reported by Warren and Sabin(5), still seem to be the accepted remedies in man(6), although in mice the carrier state and fatal relapse are usual(7). Both of these defects appear to have been avoided in an encouraging but limited number of mice treated with either p, p′ diamino diphenyl sulfone N, N′ (dextrose sodium sulfonate) (Promin, Parke Davis & Co.) or Disodium formaldehyde sulfoxylate diamino diphenyl sulfone (Diasone, courtesy of Abbott Laboratories). To the various antibiotics, arsenicals, sulfas and essential nutrients previously listed as ineffective for therapy(3,8-10), Pentaquine, Chaulphosphate, Desoxyribonucleic acid, Milibis, Aureomycin, Spleen Marrow and Sulfadiazine may be added. Biocca & Nobrega's favorable report(11) of the last named drug could not be confirmed by our trials. Sabin's strain of *Toxoplasma* has been used because no spontaneous recoveries have occurred during our 5 years of experimentation and because the survival period rarely varies (7 to 8 days following subcutaneous inoculation). Our technic for inoculations and for the administration of drugs has been reported earlier(10). No serial dilutions of the infective emulsion are made when inoculating because the nature of the parasite negates this customary estimation of virulence. An homogenous distribution of reagents particles, or parasites in the diluent is a pre-requisite for such determinations and the units of distribution in the emulsified tissue of a *Toxoplasma* infected animal consist of free parasites and the host's cells within which there may be anywhere from 0 to agglomerations of 90 parasites. That our inoculum is adequately uniform in virulence, however, is evidenced by the uniform lethality of a given inoculum. This investigation has extended over a year because long and repeated post therapeutic observations are necessary to avoid assuming asymptomatic carriers are cured animals. In the earlier experiments, only 3-6 mice were used to reduce the hazard from handling, which seems minimal since repeated trials of artificial "biting" failed to produce infections in mice (12).

Our hypothesis that toxoplasmosis is essentially (if not exclusively) a disease of the RES broadly defined(13) prompted the investigation of Promin and Diasone because these sulfones have been beneficial in leprosy, a disease with a similar predilection.

In accordance with the clinical usage of Promin(14), subcutaneous inoculations (40 mg in 0.4 ml) were made 6x a week with the interpolation of a resting week after each 2 weeks. Curative results were obtained if a "booster" injection was given midway in the resting week. One mouse from the first trial was living after 14 months (6 weeks of injections and 5 boosters). Delayed therapy in a second trial was unsuccessful but 2 of 3 mice treated within 24 hours (4 weeks of injections and 4 "boosters") were in good health after 13 months. The 3rd mouse was killed at 8 months as a donor for 5 recipients which also remained symptomless for 6 weeks and produced no symptoms when used as donors for additional recipients (Table II). Promin failed to protect in reduced dosage levels or in delayed therapy but surpassed Sulfapyridine in comparative trials. All but 1 mouse treated with 1% Sulfapyridine in the diet died after treatment stopped. The simultaneous use of Promin and Sulfapyridine

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TABLE I. Comparison of Diasone and Promin.

No. mice	No. infected	Promin, 40 mg in .4 ml	.5% diasone mg/day/mouse	Days survived
5	5	—	—	7
3	—	6x wk 6x '' Booster	—	28 Killed
5	5	6x wk 6x '' Booster	—	1 for 16, 2 for 18 1 for 19, 1 for 25
3	—	—	23.7 mg 1st 6 wk 20.3 mg 8th wk	All thrived* 1 destroyed in 32
15	15	—	17.3 mg 1st 6 wk 17.5 mg 8th wk 500 mg/kg	1 for 22 1 for 55, fighting? 1 destroyed, 32 2 donors, 44 3 donors, 60 2 challenged, 62 1 for 85 1 for 88 1 for 101 2 thriving at 130†

Therapy delayed 2½ days after inoculation.
* 1 littered at 4 wk, 6 young thrived, killed at 4 wk.
† 1 littered at 7 wk, 6 young thrived at 110 days.

gave erratic results which were surpassed by those of Diasone.

After preliminary trials demonstrated that Diasone (0.5% in the diet)(15) protected mice even when therapy was delayed, a comparison of the effectiveness of Diasone and Promin was made. As Table I shows, the 5 untreated mice died 7 days after inoculation. Survival following delayed treatment with Promin was 16 to 25 days. One escaped, Diasone-treated mouse was killed because of its questionable identity, but 13 mice were thriving and one female littered when the Diasone therapy was interrupted at 6 weeks. Two of the symptom-free, treated mice served as donors for 10 recipients which were also used as donors after 28 symptom-free days. By error, only 3 final recipients were used, but these were symptomless at 33 days (Table II). Even the nurslings thrived when Diasone was again administered during the 8th week. Interrupted therapy was used to counteract the possibility of the drug's discouraging the emergence of parasites from the supposedly resistant intracellular position. Following the 8th week, 3 symptom-free mice were used as donors for 10 recipients which all developed symptoms and died (Table II). Two symptom-free, Diasone treated mice were challenged at this time and died at 7 days, which was in agreement with results with animals treated with Sulfapyridine and Promin (Table III) and with Weinman's report that recovery from toxoplasmosis did not guarantee immunity(16).

Diasone was much more effective than Promin in protecting mice from toxoplasmosis when therapy was delayed a third of the survival time of untreated mice, but the results with Promin are of value because they surpassed those of the presently favored Sulfapyridine and are based on over a year's observation of symptom-freed mice. Diasone has not been investigated as long but appears equally capable of suppressing symptoms and eliminating the carrier condition in a rapidly fatal infection with *Toxoplasma*. Mice treated with Diasone thrived and produced and suckled healthy litters. Leprosy in man has been treated successfully with Diasone for a considerable time and the trial of this drug in therapeutically resistant cases of human toxoplasmosis in as high a dosage as is compatible with the known standards of toxicity would seem to be indicated.

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TABLE II. Investigation of Symptom-free, Treated Mice. 1. For carrier state.

Therapy	Donors		Recipients #1		Recipients #2	
	No. of mice	Description	No. of mice	Results	No. of mice	Results
Diasone	1	150 days, PI	10	NS	8	NS
	2	105 days, PI		6 donors at 28 days		Killed 47th day
	1	35 days, litter				
	2	44 days, PI	10	NS	3	NS
				6 donors at 28 days		Killed 33rd day
	3	60 days, PI	10	All dead at 12 days		
Promin	1	242 days, PI	5	NS	5	NS
				donors at 61 days		Killed 28th day
Promin and sulfapyridine	3	Newborn from mother, 91 days, PI	4	NS Killed at 28 days		—
	5	Newborn from mother, 86 days, PI	4	NS Donors at 2 mo.	4	NS Killed 28th day

PI = Post inoculation. NS = No symptoms.

TABLE III. Investigation of Symptom-Free, Treated Mice. 2. For immunity.

Therapy	No. of mice	*Toxoplasma* challenge interval	Results
Diasone	2	62 days	Dead* in 7 days (Controls in 4 days)
Promin and sulfapyridine	1	113 days and her	1 of young died in 8
	3	26 day old young	days, others in 4
	1	84 days and her	All died in 5 days
	5	20 day old young	(Controls in 4 days)
	1	247 days	Dead in 6 days
	3	214 "	Dead in 51, 67, 70 days (symptoms atypical)
	1	203 "	Dead in 6 days (Controls in 4 days)

* Preparations from ascitic fluid contained *Toxoplasma*.

Summary. In an encouraging but limited number of white mice infected with a rapidly fatal toxoplasmosis, Diasone and Promin have not only suppressed the symptoms but have repeatedly eliminated the carrier state. Symptom-freed, Promin-treated mice have survived over a year but Diasone appears to be less toxic and more potent since it protected mice when therapy was delayed for ⅓ of the survival time of simultaneously infected, untreated mice.

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Beneficial Effects of Vitamin B_{12} and Folic Acid on Recovery from Internal Radiation by P^{32}.* (18553)

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In a previous paper the toxicity of P^{32} has been investigated in relation to the content of lipotropic factors, protein, fat and phosphate in the diets(1). In a continuation of such a study, the effect of varying the supply of certain vitamins has now been tested. Damage to the bone marrow is a constant occurrence in radiation injury and should be of even greater importance after administration of radioactive compounds, such as isotopic phosphate which is deposited preferentially in the skeleton. Under this condition, one might expect that the recovery of the animals will be affected markedly by a deficiency of hematopoietic factors such as folic acid and vit B_{12}. Some beneficial effects of folic acid on the anemia and leukopenia following radiation therapy in human beings have been reported(2,3). An increase in the % of survivors and in the survival time of x-ray irradiated mice receiving generous amounts of either pyridoxine or folic acid has also been described(4). However, no significant effect of these vitamins on the hematological changes of the animals was detected. Folic acid also failed to prevent such changes after total body irradiation with x-rays of rats(5), cats(6), and swine(7), and after introduction of P^{32} in human beings, or Sr^{89} in rats(8). Likewise, vit B_{12} failed to modify the anemia and leukopenia of rats exposed to x-rays(9).

To our knowledge, no study has yet been reported concerning the effectiveness of the combined administration of folic acid and vit B_{12} on the recovery from radiation injury: a point which is directly suggested by the close relationships in the biological actions of these two compounds. Moreover, since large amounts of these and other vitamins are synthesized by the intestinal flora, an attempt to reduce such a synthesis by the ingestion of poorly absorbed antibacterial agents, would tend to give a more accurate estimation of the value of the vitamins.

Experimental. Four series of experiments were carried out on a total of 525 mice under conditions identical to those of our previous experiments(1), except as indicated below. In series I and II, 172 mice were maintained on experimental diets without added sulfasuxidine and injected with 6 μc/g of P^{32}. Large doses of folic acid (20 mg/100 g of diet) or of a mixture of folic acid (20 mg/100 g) and vit B_{12} (0.75 mg) were added to the diet of part of these animals. In Series III and IV the low fat, low protein diet (Diet No. 34) (1) contained 5% sulfasuxidine and a basal mixture of B vitamins (Thiamine HCl 0.5 mg, riboflavin 0.5 mg, pyridoxine HCl 0.5 mg, Ca

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TABLE I. Effects of Certain Vitamins on Survival of Mice Injected with P^{32} (5 μc/g) and Maintained on a Sulfasuxidine Containing Diet.*

Supplements	No. of mice	Avg food consumption, g/day	Avg change in body wt, g	Time of 50% deaths days	% of survivors 21st day	% of survivors 56th day	Avg time of survival,† days
0	73	3.5	—1.6	27	58	16	29 ± 2.0
FA	39	4.1	+5.0	20	49	31	30 ± 3.3
B_{12}	24	3.7	—3.8	34	58	46	36 ± 4.0
FA + B_{12}	34	4.1	—0.1	>56	76	56	42 ± 3.5
FA, B_{12}, K, Bio.	67	4.0	+1.1	54	72	49	41 ± 2.5
0‡ (controls)	18	4.1	—6.7	>56	100	83	55 ± 0.8

* Diet No. 34(1) containing 5% sulfasuxidine. A basal B vit. mixture (see text) was incorporated daily in the diet.

† In the calculation of the avg time of survival, a survival time of 56 days was ascribed to the mice still alive at the end of the experiments. Values preceded by ± are the standard errors of the means.

‡ Not injected with P^{32}.

TABLE II. Statistical Comparison of the Survival of Mice Injected with P^{32} (5 μc/g) and Maintained on a Sulfasuxidine-containing Diet.

Supplements	Avg time of survival n	Avg time of survival t	Avg time of survival P	No. of survivors at 56th day χ^2	No. of survivors at 56th day P
FA	110	0.23	>.05	2.32	>.05
B_{12}	95	1.67	>.05	7.56	<.01
FA + B_{12}	105	3.19	<.01	15.70	<.01
FA, B_{12}, K, Bio.	138	3.85	<.01	15.92	<.01

* n = Degrees of freedom; t = According to Fisher(10); P = Probability for a chance occurrence.

pantothenate 2 mg, inositol 2 mg). In addition, some groups received also, as "supplements" to each 100 g of the diet: folic acid alone (FA = 1.5 mg), or vit B_{12} alone (B_{12} = 0.45 μg), or both folic acid and B_{12} (FA + B_{12}; 1.5 mg and 0.45 μg, respectively), or a mixture (FA,B_{12},K,Bio) of folic acid (1.5 mg), vit B_{12} (0.45 μg), 2,methyl-1,4-naphthoquinone (Menadione, 15 μg), and biotin (1.2 μg). Three groups of 6 mice each were maintained for the whole period of the experiments on the sulfasuxidine-containing diet without supplements and were not injected with the isotope. All other animals were maintained on the diets, unsupplemented or supplemented, for 10 days before the introduction of P^{32}, and for 56 more days thereafter. Each of these mice received a single injection of P^{32} (5 μc/g): from our previous data this dose was estimated to be slightly below the LD_{50} (at the 21st day and for a low-protein, low-fat diet)(1). In the experiments of Series IV, one animal in each group was sacrificed by decapitation immediately before and at regular intervals after the introduction of P^{32}, so that blood could be obtained for determinations of the hemoglobin content, red blood cell and white blood cell counts, and hematocrit.

Results. In the experiments of Series I and II the diets did not contain sulfasuxidine, and therefore notable amounts of vitamins were probably still available to the animals through synthesis in the intestine. Supplementation of such diets with folic acid, or folic acid and vit B_{12}, did not appear to cause any significant effect on the survival of mice injected with a dose of P^{32} in the higher range of the LD_{50} (at the 21st day)(1). Data of the experiments of these series (and of Series IV also) are omitted for brevity. Only the data on the survival of mice on the diets containing sulfasuxidine and the results of a statistical treatment of these data are recorded in Tables I and II, respectively. A few of

10. Fisher, R. A., *Statistical Methods for Research Workers*, London, 1936, 6th Edition.

the mice not injected with P^{32} died after several weeks on the low protein diet with added sulfasuxidine. On the other hand, in the groups of mice injected with 5 μc/g of P^{32}, the % of survivors at the 21st day and the time of 50% deaths were the same as, or greater than the values previously observed in mice injected with a smaller dose of P^{32} (4 μc/g) and maintained on a diet without added sulfasuxidine (Diet 31, which differed from Diet 34 only in a higher fat content) (1). It seems therefore that the inclusion of sulfasuxidine in the diet did not enhance the susceptibility of the animals to the injurious action of the isotope.† Supplementation of the sulfasuxidine-containing diet with folic acid alone increased the % of survivors at the 56th day, but the increase was not statistically significant ($P>0.05$). Vit B_{12} seemed more effective, however only the increase in the % of survivors was significant. On the other hand, the combined administration of folic acid and vit B_{12} offered a marked protection with a high degree of significance for both % of survivors and survival time. The further addition of menadione and biotin did not enhance the beneficial action of the mixture of folic acid and vit B_{12}. Altogether our results suggest that none of the vitamins tested modify appreciably the damage caused by the introduction of P^{32}, but that an adequate supply of both vit B_{12} and folic acid may be an important factor for the recovery of the damaged tissues.

The hematological changes following the introduction of P^{32} in mice on the sulfasuxidine-containing diet showed approximately the same general behavior in all groups of Series IV. After an initial fall, the values rose progressively and often declined again at the longest intervals. However, between the 12th and the 36th day, that is, during the recovery period after the initial fall, the % hemoglobin and the red and white cell counts in the blood of mice on the diet supplemented with both folic acid and vit B_{12} were all higher than in the other groups. Because of the limited number of determinations, the differences observed can only be taken as a suggestive indication for a relationship between the improved hematological picture and the prolonged survival of mice receiving the mixture of the two vitamins.

Summary. The addition of generous amounts of folic acid, or folic acid and vit B_{12}, to experimental diets had no significant effect on the survival of mice injected with a dose of P^{32} in the higher range of the LD_{50} (21st day). On the other hand, when sulfasuxidine was added to the diet and the mice were injected with a dose of P^{32} slightly below the LD_{50} (21st day), the administration of vit B_{12} and folic acid increased significantly the time of 50% deaths, the average time of survival and the % of survivors (at both the 21st and the 56th day).

† Several recent reports suggest that antibiotics may even be beneficial in the treatment of radiation injury by preventing general or localized infections. For example, streptomycin and penicillin effectively reduced the mortality or prolonged the survival of rats injected with P^{32}(11).

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Development and Secretion of the Blood Group Factor O in the Newborn. (18554)

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As part of a series of investigations on the development and secretion of blood group properties, we have investigated the development and secretion of the O property in the newborn, child, and adult. The development of the O property has been studied by determining the agglutinability and group-specific absorbing power of red blood cells, using

an anti-*Shigella dysenteriae* immune goat serum. This serum was absorbed by mixing one volume of the serum with three volumes of saline and one volume of packed washed A_1B cells; the mixture was allowed to stand in the refrigerator for 30 minutes, and the serum was separated by centrifugation. The absorbed serum gave strong positive reactions with all O and A_2 cells, and only weak reactions with a certain percentage of bloods of group A_1 and B, in conformity with Hirszfeld's theory. The same serum in a 1:8 dilution reacts exclusively with group O and group A_2 blood cells. These observations were made in tests on a series of approximately 4,000 blood specimens.

The absorbing power was determined by incubating for one hour at 37°C the serum diluted 1:4 in saline solution with one-fourth volume of washed, packed red cells; the absorbed serum was titrated against standard group O test cells from an adult. As a control, parallel absorption experiments were carried out with a standard specimen of group O red blood cells. In all the experiments the red blood cells of the same group O adult, with which the anti-O serum was titrated, were always used as controls. The blood to be tested was almost always obtained by venipuncture; except in young children when it was obtained at times from the heel. The tests were carried out on 75 newborn infants (from the Obstetrical and Gynecological Clinic of the University of Pavia) on their first and second days of life, and repeated after a fortnight. Blood samples were also collected from 100 children (Pediatrics Clinic of the University of Pavia) of ages ranging from a few days to 7 years all of whom were either healthy or had no blood disease. In addition, blood samples were collected from 50 individuals of ages 10 to 30 years, from students, blood donors, and other healthy persons. The tests on the children and adults were repeated 3 to 5 times at intervals of at least one week. In the younger children (one to 12 months of age) the tests were also made at intervals of 6 months. In this way it was

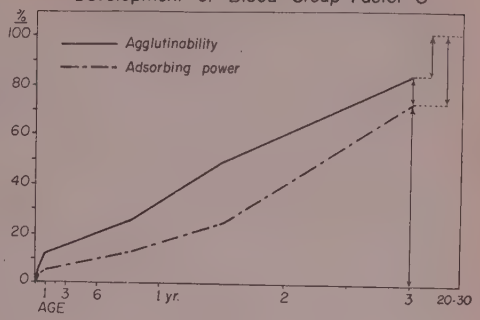

FIG. 1.

possible to demonstrate the development of the O agglutinogen in the same individual as well as in a random group of newborn and adults. The O substance in the saliva was determined with the same anti-*Shiga* immune goat adsorbed serum, diluted with 32 volumes of saline solution (*i.e.*, at 1:8 titer) by a quantitative method(1) and using progressively doubled dilutions of the saliva, collected from newborn infants and very young children with a thick cotton swab rubbed over the floor of the mouth.

The results were fairly uniform for each of the different ages and may be summarized as follows (Fig. 1): the agglutinability of group O blood cells of newborn infants and of infants during the first days of life, determined by an anti-O serum (goat anti-*Shiga* immune serum absorbed with A_1B blood cells) shows values which may vary from 1:4 to 1:16 (control tests with adult O blood cells gave titers up to 1:256); only exceptionally were titers against newborn cells higher. The agglutinability increases progressively with age, and approaches adult values after one to 2 years; at the age of 5 years values identical with those of adults were observed.

The group-specific absorbing power has been found to be considerably lower than in adults, and correlated with the agglutinability. The absorbing power also increases progressively with age, in parallel with the agglutinability. These results are in conformity with

1. Formaggio, T. G., *Min. Medicoleg.*, 1950, v70, 103.

2. Formaggio, T. G., *Atti 3rd Congr. Internaz. Transf. Sangue,* Torino, 1948, v1, 185.

those found(2) when investigating the development of the agglutinogens A and B in the newborn and infant.

The amount of O substance in saliva as determined by its capacity to neutralize the anti-O serum has been found approximately equal to that of saliva of adults of the "secretor" type; hence, it may be concluded that there is no direct proportional relation between the quantity of antigen present in the red blood cells and the quantity of antigen present in the body fluids. The relative frequency of the secretor and non-secretor types is the same in newborn, in children, and in adults of O group.

If one may assume without qualification that our serum reveals the existence of a special antigen peculiar to the blood group O, our results could be interpreted in the same way with those obtained with the agglutinogens A and B [Kemp(3), Morville(4), Thomsen and Kettel(5), Formaggio(2)]. Recent reports by Boorman, Dodd and Gilbey(6), by Morton and Watkins(7), by Grubb(8) and others, on the nature of the O substance and on the heterogenetic antigen "H," however, suggest that our experimental results should perhaps be interpreted more cautiously.

From our own experimental data it seems that the supposed essential difference between O and H antigens cannot be considered as proved; moreover, it cannot be denied that animal anti-O sera (and in particular the goat anti-*Shiga* immune serum) show group O specificity, and not merely a generic H specificity. It may be presumed that anti-O sera do not contain a single specific antibody, but contain a complex mixture of antibodies of different specificity, the proportions of which vary in sera of different origin, and this may account for the different results obtained by various workers. Among these antibodies there is undoubtedly at least one which is truly O specific (perhaps related to the product of Bernstein's gene *O*). It is probably with such anti-O sera that the behavior of agglutinogen O of red blood cells, analogous to that of agglutinogens A and B, is best demonstrated.

Summary. The development of the property O in the newborn and in young children, investigated from the point of view of the agglutinability and group-specific absorbing power with an immune anti-*Shigella dysenteriae* goat serum, is comparable to that of the agglutinogens A and B, namely, the agglutinability and the absorbing power are both extremely poor at birth, but increase until after 3 to 5 years their values become equal to those of the adult. Saliva from newborn and children of group O of the "secretor" type, neutralizes anti-O serum approximately to the same degree as saliva of adults of group O. The results obtained seem to contradict recent investigations which consider the so-called anti-O sera to have only an anti-H specificity. Probably, anti-O sera contain a mixture of serologically related antibodies to some of which cannot be denied an anti-O specificity *sensu strictiori.*

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Color Test to Measure the Toxicity of Adrenal Cortex Hormones to Lymphocytes.* (18555)

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Two methods of studying the cytotoxicity of reagents have been presented in previous reports—the method of unstained cell counts (1) and a color test(2). The method of unstained cell counts is based on the property of living cells to resist staining with such dyes as safranine and eosin. By the addition of safranine, the number of living, *i.e.* safranine resistant, cells can be determined in a suspension before and after treatment with a reagent. This method was used to measure the toxicity of adrenal cortex hormones and other reagents. The color test is based on the capacity of a cellular suspension to reduce the oxidation-reduction indicator, 2,6 dichlorophenol indophenol, even when the suspension is exposed to air. The reduction of the dye was inhibited when the toxic agent, formaldehyde, was added to the suspension.

The question now arises whether the toxicity of adrenal cortex hormones to lymphocytes can be demonstrated and measured by means of the redox indicator.

Cytotoxicity of lipo-adrenal cortex. Cells of the rabbit thymus were suspended in a medium of serum and Ringer solution. The reagent, Lipo-Adrenal Cortex,† and the indicator, 2,6-dichlorophenol indophenol, were added to the suspension. The mixtures were incubated at 37°C in small test tubes which were shaken mechanically to prevent settling of the cells. In one hour the blue color of the test mixtures disappeared as a result of the reduction of the indicator. After about 7 hours of incubation, a slight blue color reappeared in the mixtures containing Lipo-Adrenal Cortex but not in the control suspensions without reagent. After 24 hours, the treated suspension had a distinctly blue color but the control suspension had only a trace of color. The differentiation in the intensity of color was sharp and definite. This experiment indicated that the Lipo-Adrenal Cortex had an inhibiting action on the reduction of the indicator by the cells. This inhibiting action was readily obtained with a low concentration of reagent (0.00,004 ml of the extract or 0.00,16 rat unit in the 0.4 ml of test solution). Furthermore, it was evident that the lipoid preparation had no immediate toxic effect but produced its first appreciable action after about 7 hours of incubation.

Titration of Lipo-Adrenal Cortex. The suitability of the redox test for the titration of adrenal cortex extracts and hormones was studied in a few preliminary experiments. A standard mixture was first prepared which consisted of equal parts of fresh and heat inactivated (56°C 1 hour) suspension plus a small amount of indicator. This mixture, evidently, contained one-half the number of living cells as the fresh suspension. The test preparations consisted of the fresh suspension with variable amounts of Lipo-Adrenal Cortex and with indicator. After 24 hours of incubation, the colors of the various mixtures were compared to determine which test preparation had the same intensity of color as the standard. This test preparation determined the titer of the reagent. In experiments on Lipo-Adrenal cortex, the suspension used contained 200,000 living cells per cubic milli-

* Published with the approval of the Chief Medical Director, Veterans Administration. The statements and conclusions published by the author are the result of his own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

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† Lipo-Adrenal Cortex is a commercial preparation of The Upjohn Co. It is a purified and concentrated extract, in cottonseed oil, of the adrenal cortex. Each milliliter is reported to contain 40 rat units of cortical activity and to be equivalent to 1 mg of 17-hydroxycorticosterone according to the liver-glycogen deposition test.

TABLE I. Abstract of an Experiment to Determine the Titer of Lipo-Adrenal Cortex (Lac).

	Test mixtures				
Tube No.	Fresh-suspension, ml	Heated suspension, ml	Redox indicator 1:10000 ml	LAC rat units in .04 ml serum added to tubes	Intensity of color after incubation 37°, 24 hr
A	.32	0	.04	0	±
B	.16	.16	.04	0	++
C	0	.32	.04	0	+++
D	.32	0	.04	.0004	+
E	.32	0	.04	.0008	++
F	.32	0	.04	.0016	+++

Mixture in tube E had same intensity of color as tube B with 50% fresh suspension. The titer is, then, .0008 rat unit of lipo-adrenal cortex.

meter. The final concentration of indicator was 1:100,000. Under these conditions it was found that Lipo-Adrenal Cortex in a dilution of 1:20,000 produced a color equal to that obtained with a mixture of 50% fresh suspension. The titer of the commercial extract was then approximately .00,08 rat unit in the 0.4 ml of test fluid. The method used for the titration of the extract is exemplified in Table I.

The titration of the reagent has to be done with strict aseptic technic. The contamination of the suspensions with bacteria will usually cause the complete reduction of the dye and may cause the death of the lymphocytes. The color test appeared to be a simple and convenient method for the determination of the approximate titer of an adrenal cortex preparation. For a more accurate titer it would probably be necessary to use a pure adrenal cortex hormone as a standard and to use spectrophotometric methods to measure the color.

Cytotoxicity of other adrenal cortex steroids. Toxic effects, *i.e.* inhibition of the reduction of the indicator in the redox test, were obtained with 1 γ of cortisone acetate (Cortone, Merck). In contrast, 200 γ of desoxycorticosterone (Cortate, Schering) gave a negative test. The present findings with the color test and the previous observations by means of unstained cell counts are in accord. Both the color and the counting method showed that Lipo-Adrenal Cortex and Merck's Cortone are toxic but that desoxycorticosterone is not toxic to cellular suspensions derived from the rabbit thymus.

Specificity of the redox test. A positive redox test may be defined as an inhibition of the reduction of the indicator by a suspension treated with a reagent as compared to a control suspension without reagent. Positive tests were obtained with many reagents including formaldehyde, x-rays (100r) and nitrogen mustard (0.4 γ). Simultaneous viable cell counts indicated that these reagents in the doses tested were cytocidal to the lymphocytes. The color test was then not specific for adrenal cortex hormones but was positive with all of the toxic agents tested. As would be expected a positive redox reaction was also obtained with the oxidizing agent, potassium ferricyanide (0.001M). The reagent was, however, not toxic to the cells according to the method of unstained cell counts. Evidently a positive test does not by itself prove that a reagent is cytocidal. On the other hand, the reduction of the indicator by a cellular suspension does not necessarily indicate that the cells in the suspension are viable. The accidental presence of bacteria and other extraneous reducing agents in the suspension will decolorize the dye. In this connection, it may be noted that a cell in dying may liberate reducing agents. There may then be a lag time between the death of the cells and their loss of the ability of cells to reduce the dye.

These limitations in the use of the redox indicator as a measure of the number of viable cells does not invalidate the color test but does indicate the precautions needed in interpreting the results. Under the proper conditions, the color test provides a means of titrating the toxicity of extracts of the adrenal cortex.

Summary. Suspensions of living lympho-

cytes decolorize the oxidation-reduction indicator, 2,6 dichlorophenol indophenol. This reduction was inhibited by the cytotoxic agents, lipo-adrenal cortex, cortisone, nitrogen mustard and x-rays but was not affected by desoxycorticosterone. The method was used to titrate lipo-adrenal cortex, the titer being .00,08 rat unit. This color test seemed to be a convenient method for demonstrating and measuring the toxicity of the adrenal cortex hormones to lymphocytes.

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Action of Various Hormones on the Spread of Subcutaneously Injected Hemoglobin.* (18556)

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The spread of a subcutaneously injected dye is dependent on numerous factors, the most important being Duran-Reynals' spreading factors(1). The hyaluronidase acting on hyaluronic acid in the mesenchymal ground substance is the best known but it is beyond doubt that apart from hyaluronidase, numerous enzymatic and non-enzymatic factors are involved in the spread mechanism. Hechter(2) has shown recently that spread is dependent upon various factors such as edema, trypsin, peptones and so forth. The mechanism of their action is not yet clarified. It seems to be either correlated with an increase of the local hyaluronidase secretion by the non-enzymatic injected spreading factor, or explained by alterations of non-hyaluronic components of the dermal barrier such as chondroitin sulfuric acid(3,4). Hormonal control of the spread was shown by Menkin (5) and Opsahl(6,7). They stated that adrenal secretions were related to the spread regulatory mechanism.

FIG. 1.

The purpose of this work was to control

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† Fellow of the Swiss Academy of Medicine and Biology.

‡ Fellow of the Canadian Life Insurance Officers Assn.

§ UNESCO Fellow (Canada).

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the spread of an hemoglobin spot in the skin of the rat, under standard conditions of treatment with various hormones or hormone combinations.

Material and methods. Seventy-five piebald, female rats of an average initial body weight of 150 g were divided into 11 groups. The animals of the first group were used as controls. The rats of the other groups received separate daily injections of 5 mg of desoxycorticosterone acetate (DCA), 10 mg of methyl testosterone, 0.5 mg of thyroxine, 5 mg of cortisone, 3 mg of somatotrophic hormone (growth hormone or STH), 40 mg of lyophilized anterior pituitary (LAP) and the following combinations at the same dosage level: LAP and DCA, STH and cortisone, methyl testosterone and cortisone, thyroxine and cortisone. After 10 days of treatment 0.1 cc of a 15% solution of hemoglobin was injected into the posterior region of the back, which area had been depilated the previous day. The spot was measured two and one half hours later. The area was calculated by the following formula:

$$S = \pi \frac{\text{larger diameter} \times \text{smaller diameter}}{4}$$

Results. As seen in Fig. 1, cortisone significantly inhibits the spread while DCA, STH, LAP and thyroxine enhance it. Testosterone has no notable action. In the association of cortisone with STH and thyroxine, the spread inhibiting effect of cortisone prevails over the enhancing action of these compounds. This property is less pronounced when cortisone is combined with LAP. The enhancement observed following concurrent treatment with DCA and LAP represents the sum of their separate actions.

Summary and conclusions. The spread of an hemoglobin spot was studied under various hormonal conditions. At the dose level utilized, cortisone antagonizes the enhancement of the spread normally occasioned by STH, thyroxine and LAP. Our results confirm the antagonism between DCA and cortisone first suggested by Selye(8) concerning their action on vessels and inflammation. LAP, STH and thyroxine in this respect have a DCA-like action opposite to the effect of cortisone.

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Failure of ACTH to Protect Against Acutely Lethal Toxins of Influenza Virus and Rickettsiae.* (18557)

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The capacity of adrenocorticotropic hormone (ACTH) to alter the clinical course of certain infectious diseases(1-3) suggests the need for investigation of the mechanisms involved in these changes. One of the possible explanations of the effect of ACTH in clinical infections is that the adrenal cortical hormones aid in the detoxification of the noxious products of the infectious agent. In order to test this hypothesis, the effect of ACTH

* Aided by grants from the U. S. Public Health Service.

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on the acute toxic action of concentrated suspensions of influenza virus and of rickettsiae has been investigated using doses of the hormone which have been demonstrated to produce prolonged eosinopenia in mice(4) and rats(5).

Materials and methods. ACTH§ was diluted in saline and 0.5 ml containing 2.5 mg was injected intraperitoneally into mice every 12 hours(4). The rats received 2 mg subcutaneously every 6 hours. The PR8 strain of influenza virus was used throughout; the method of obtaining and administering the virus has been described(6). Infected allantoic fluids were concentrated by adsorption on and elution from chicken erythrocytes and were injected intravenously into mice or rats. Concentrations of virus were such that 1.5 ml per 100 g of body weight killed about 70% of the mice within 24 hours and uniformly killed rats in 9-12 hours. In the strain of rats used, the time of survival after intravenous injection of the virus was roughly inversely proportional to the dose; twice the dose employed was fatal in 4-6 hours while rats receiving half that amount survived up to 24 hours and still smaller doses permitted increasing rates of survival(5). The rickettsiae used were the Wilmington strain of murine typhus *(R. mooseri)* and the Breinl strain of epidemic typhus *(R. prowazeki)*. Infected yolk sacs were pooled and diluted in a buffered salt solution containing sucrose and glutamate (7). The toxicity of the rickettsial suspensions was determined by injecting 0.25 ml of the test suspension into the tail veins of lightly anesthetized mice. Four to 6 mice were used for each dilution and mice dying within 24 hours were considered to have succumbed to the acute toxic effect of the rickettsiae. Half-logarithmic dilutions were titrated in this manner and the LD_{50} of a given preparation of rickettsiae was calculated by the method of Reed and Muench(8). Experience with this method of testing acute toxicity of rickettsiae in mice has shown that differences in the LD_{50} greater than 3-fold (approximately 0.5 log dilutions) may be considered significant(9). The infectious agents were stored at −70°C between tests. The mice were white Swiss mice of the Tumblebrook Farm strain weighing 10-14 g for the rickettsial experiments and 15-18 g for the influenza virus experiments. The rats were males of the Sprague-Dawley strain weighing 110-125 g. Immune animals received sublethal doses of the influenza virus 2-4 weeks before they were challenged.

Experimental. ACTH was given to 30 mice

TABLE I. Effect of ACTH on Toxicity of Influenza A Virus to Mice.

Test substance inj.	No. of inj.* prior to challenge	No. dead / No. inj.
ACTH	3	10/10
	5	7/10
	7	10/10
Saline	3	7/10
	7	7/10
0 (immune)	—	0/6

Each animal received 1.5 ml of virus concentrate per 100 g body wt intrav.

* ACTH dosage was 2.5 mg in 0.5 ml intraper. every 12 hr until termination of experiment. Saline was given in 0.5 ml amounts every 12 hr.

TABLE II. Effect of ACTH on Toxicity of Influenza A Virus to Rats.

Test substance injected	Result				
ACTH*	9	9	9	9	10†
Saline	9	9	9	9	9
0 (immune)	S	S	S	S	S

Each animal received 1.5 ml of virus concentrate per 100 g of body wt intrav.

* ACTH dosage was 2 mg in 0.5 ml subcut. every 6 hr until death. Saline dosage was 0.5 ml subcut. every 6 hr.

†Hr after virus was inj. when death occurred.

S = Survived.

Experimental. ACTH was given to 30 mice

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TABLE III. Effect of ACTH on Toxicity of Typhus Rickettsiae for Mice.

Rickettsial strain	Dilution of rickettsial suspension†	Results of challenge* in groups of mice prepared by			
		Saline‡	Bovine serum albumin‡	ACTH‡	No treatment
R. prowazeki (Breinl)	1.0	—	5/5	5/5	4/4
	1.5	—	4/4	5/5	4/4
	2.0	—	0/5	3/6	1/4
	2.5	—	0/4	0/4	0/4
	LD_{50}§		1.7	2.0	1.8
R. mooseri (Wilmington)	1.4	5/5	4/4	4/4	4/4
	1.9	5/5	3/5	6/6	5/5
	2.4	0/6	1/6	0/6	1/4
	2.9	0/4	0/4	0/4	0/4
	LD_{50}§	2.1	2.0	2.1	2.2

* Ratio: No. of mice died/total mice in group.

† Expressed as log of denominator of the dilution. Each mouse received intrav. 0.25 ml of stated dilution of yolk sac suspension of typhus rickettsiae.

‡ Mice received 3 doses intraper. at intervals of 12 hr in a volume of 0.5 ml for each dose; saline = 0.85% NaCl; bovine serum albumin = 5 mg per ml; ACTH = 5 mg per ml.

§ Expressed as the log of denominator of the dilution of the LD_{50}.

and groups of 10 of these mice were injected with influenza virus after the 3rd, 5th and 7th injections of hormone. Twenty control mice were given 0.5 ml of saline every 12 hours; half of them were given the virus after the 3rd, and the remainder after the 7th injection of saline. Table I shows that ACTH did not increase the survival rate of the mice dying of the acute toxic effects of influenza virus. Immune mice all survived the challenge.

Five rats were challenged with influenza virus after the second dose of ACTH had been given and 5 control animals which received the same volume of saline every 6 hours were challenged in a similar manner. Injections of ACTH and saline were continued until death of the animals had occurred. ACTH did not increase the survival time of the rats (Table II).

The rickettsiae were injected after the third dose of ACTH had been administered to the test mice. Control animals included groups which received injections of saline (0.5 ml) or bovine serum albumin (2.5 mg in 0.5 ml) every 12 hours. ACTH did not significantly affect the mortality due to rickettsial toxemia (Table III).

Discussion. Adrenalectomized animals are more susceptible than intact animals to many toxic substances and this increased susceptibility is alleviated in part or entirely by adequate doses of adrenal cortical extract(10). However, resistance to toxemia has been difficult to produce in intact animals despite the use of large doses of adrenal cortical extract, although there are conflicting observations on this point(10). There is evidence that neither ACTH nor cortisone inhibit bacterial or viral growth and there are indications that cortisone may actually aggravate infections in some instances(11-15). While the experiments reported in this paper were in progress, Jackson and Smadel(16) reported their failure to demonstrate any protective effect from ACTH or cortisone on the toxicity of *R. tsutsugamushi, R. prowazeki* and *Salmonella typhosa* in mice. However, the doses of hormone which they used were very much less

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than those used in the present experiments and were probably inadequate for the production of sustained eosinopenia(4).

The failure of ACTH to reduce the acute toxic effect of influenza virus and of rickettsiae as well as the failure of the hormone to increase the survival rate of mice with pneumococcal and influenza viral infections(11,17,18) leaves unexplained the mechanism by which the dramatic changes in the clinical course of pneumococcal and viral pneumonias have been produced. It is possible that such changes represent relatively secondary phenomena reflecting such effects of ACTH as its antipyretic action(19). It appears unlikely that the clinical improvement which follows the administration of ACTH to patients with infectious diseases is due to stimulation of the capacity of the host to resist the acute toxic action of the infectious agent.

Adrenal cortical hormones are not essential to the metabolism of cells and it is conceivable, therefore, that the toxic agents tested in these experiments interfere with cellular metabolism at levels of enzymic activity which are not directly under regulation by adrenal cortical hormones.

Summary. ACTH did not reduce the acute toxic effect of influenza virus in mice or rats nor the similar action of the rickettsiae of murine and epidemic typhus in mice.

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Development of Thyroid Neoplasms in the Rat Following a Single Injection of Radioactive Iodine.* (18558)

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In recent reports(1,2) we called attention to the presence of atypical epithelial cells in the thyroid glands of rats 8 months after they had been injected with single large doses of I^{131}. These cells resembled the so-called Hürthle cells found in both neoplastic and non-neoplastic thyroid glands of man. These cytological changes led us to extend our studies to later intervals.

The present report deals with 10 rats that were sacrificed 18 months after I^{131} treatment. Neoplastic changes were observed in the thyroids of two animals. In addition, Hürthle-like elements were prominent in all thyroid glands.

Experimental. Ten male rats of the Long-Evans strain, which had received a single intraperitoneal injection of 400 μc of I^{131} 18 months earlier, were anesthetized with ether and exsanguinated. The tracheas with attached thyroid glands were removed and fixed in 10% neutral formalin, embedded in paraffin, and sectioned serially at 10 μ. Representative sections of liver and kidney were sectioned at 6 μ. All tissues were stained with hematoxylin and eosin. Wilder's stain was employed for the demonstration of reticulum.

Thyroids of rats 1-8. Microscopic examination of the thyroid glands of 8 of the rats revealed the typical post-radiation picture of fibrosis, fibrosed and hyalinized vascular channels, and glandular atrophy. These changes were similar to those described in our earlier

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† Atomic Energy Commission Fellow.

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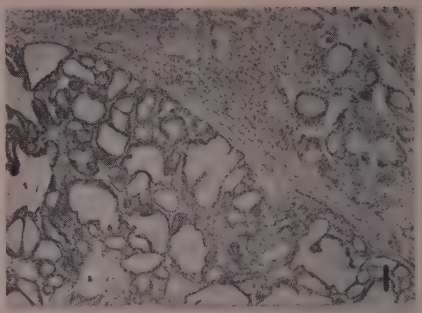
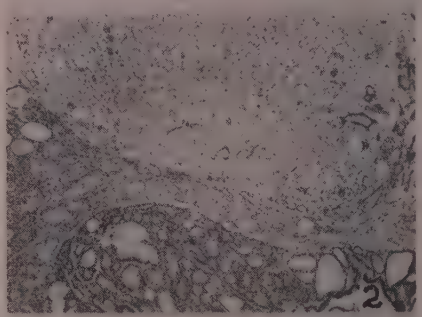
communication(1). Large areas containing follicles filled with pale colloid were found in all thyroid glands. The lining cells of the follicles were, for the most part, squamous or low cuboidal, and their nuclei showed no abnormalities. The cytoplasm of some of the epithelial cells contained a yellow granular material, identification of which was not attempted. Abundant fibrous tissue pervaded the glands, effecting wide follicular separation and appearing to compress the glandular and vascular elements. Many scattered, single epithelial cells were observed in this intracinar fibrous tissue. These cells contained a pale cytoplasm, and were quite irregular in shape. Many of their nuclei were extremely large and exhibited marked pleimorphism. The chromatin content of the nuclei was meager. In some areas a slight, chronic inflammatory condition persisted. This consisted of large and small lymphocytes, macrophages, plasma cells, and many tissue eosinophils. A constant feature in the parenchyma of all glands was the widespread occurrence of large, irregular epithelial cells (Hürthle-like) containing intensely eosinophilic, granular cytoplasm, and large, pleiomorphic nuclei which varied greatly in chromatin content and intensity of staining. These cells were scattered throughout the gland either singly or in groups; occasionally they formed small follicles with deep eosinophilic colloid. Collections of these cells, arranged in clusters or cords, were found principally at the poles of the glands.

Thyroids of rats 9 and 10. The thyroid glands of these 2 rats were composed of altered parenchyma, nodular areas, and, in addition, normal appearing tissue. The parenchymatous changes described for rats 1-8 were also observed in rats 9 and 10. In the former three neoplastic thyroid foci were noted; in rat 10, four foci were found in one lobe and 3 in the other lobe. Surprisingly enough, all tumors present in a single lobe were not of the same type. Multiple adenomata, consisting of large and small follicles lined by epithelium, ranging from cuboidal to high columnar, and containing a thin, pale, granular eosinophilic colloid, were found in both animals (Fig. 1, 2). The nuclei were quite regular and hyperchromatic. Cystic and hemorrhagic changes had occurred. A





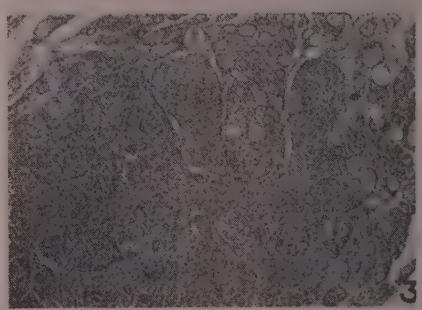


FIG. 1. Benign adenoma found in thyroid of rat No. 10. Note slight encapsulation and lack of colloid in follicles of adenoma. Normal thyroid tissue is seen in lower left corner. Hematoxylin and eosin ×34.

FIG. 2. Two tumors found in lobe of thyroid of rat No. 9. Normal tissue is present between the tumors. The tumor at upper right is that designated type A in the text. Note the poor encapsulation of this tumor and only occasional acini are seen in an almost solid sheet of cells. The tumor at lower center is a benign adenoma. Hematoxylin and eosin ×34.

FIG. 3. Portion of another tumor (type B) found in the thyroid of rat No. 10. Hematoxylin and eosin ×34.

thin, fibrous capsule separated these tumors from the surrounding tissue, although in some areas the encapsulation was not complete. These adenomata, which exerted marked compression on the adjacent normal tissue, did not appear malignant as judged by morphologic criteria. Two distinct types of thyroid tumors (A and B) that bore no resemblance to the adenomata described above were also observed in these rats.

Type A. This tumor which is shown in Fig. 2, was found in both rats. It was composed of sheets of large, irregular polyhedral cells that stained weakly eosinophilic and that contained a granular cytoplasm. Only occasionally were these cells arranged in acini. The nuclei were chromatin-poor and stained quite weakly. Mitotic figures were noted. This tumor was only partly encapsulated by a few strands of fibrous tissue.

Type B. This type of tumor, which is shown in Fig. 3, was found only in rat 10. It was composed of small, tightly packed cells containing pleiomorphic and hyperchromatic nuclei. *This neoplasm was invading adjacent tissue and showed no evidence of encapsulation.* Mitotic figures were frequent. A reticular stain revealed sparse argyrophilic fibers. The amount and distribution of reticulum left no doubt that this lesion was not of a sarcomatous nature.

Examination of the livers and kidneys revealed no evidence of metastases. No pathologic alterations were observed in the livers of any of the animals. The kidneys in 2 of the animals revealed some glomerulosclerosis, interstitial scarring, and chronic interstitial nephritis.

Discussion. It is necessary to call attention to the 2 types of tumors designated here A and B. Although we recognize that a diagnosis of malignancy is difficult to make—particularly in the case of the thyroid—without evidence of metastatic invasion, the possibility that these tumors may be malignant should be tentatively entertained for the following reasons: (1) their morphological resemblance to malignant thyroid lesions (type A to adenocarcinoma and B to small cell carcinoma) seen in man; (2) their lack of encapsulation; and (3) their invasion of neighboring thyroid tissue (Fig. 3). Further studies directed, in particular, to the question of their malignancy are in progress in this laboratory.

A review of the literature shows that spontaneous thyroid carcinomata rarely occur in the rat. Bullock and Curtis(3) state "of the many thousands of rats which have been examined post mortem only two developed thyroid tumors." One was a benign adenoma; the other, a malignant growth, was found in a rat 948 days old. Van Dyke(4) found benign thyroid adenomata only in a group of very old rats (801-906 days old). In this laboratory, examination of well over 1,000 rats of various ages up to 2.5 years failed to reveal thyroid malignancy(5). Moreover, in rats that were fed a diet containing 0.2% propylthiouracil for various periods up to 28 months, we found only occasional adenomata. Moon examined serial sections of thyroids of 14 rats, 615 to 723 days old, of the same strain as that used in the present study(6). He found no evidence of malignancy but he did observe 6 small adenomata. Thus, there can be little doubt that the 2 malignant-like thyroid neoplasms found among the first 10 rats we sacrificed 18 months after I^{131} treatment were not of spontaneous origin but were related to the administration of radioactive iodine.

The mechanism underlying the production of the tumors described here is, as yet, not clear. Two possibilities will be considered. Overstimulation of the thyroid epithelium by excess thyrotrophic hormone is believed to be a factor in the genesis of thyroid tumors. For example, malignant thyroid disease in man, according to Joll(7), is common in endemic goiter areas. Experimental work on the rat also indicates that the major etiological factor in the genesis of thyroid tumors is chronic overstimulation of the gland by thyrotrophic hormone(8,9). The Hürthle-like cells found

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throughout the thyroid parenchyma of all rats may also be indicative of exhaustion from excessive thyrotrophic stimulation.

The possibility that the trapped I^{131} might initiate neoplastic changes in the thyroid gland has been recognized by many clinical investigators(10-13) who have expressed the need for caution in the use of radioiodine for diagnosis and treatment of Grave's disease. The discovery of 2 highly suspicious tumors among the 10 rats that were treated with radioactive iodine further emphasizes the need for caution.

Summary. Examination of 10 rats sacrificed 18 months after a single injection of 400 μc of radioactive iodine revealed the following changes in the thyroid gland: 1. Hürthle-like elements (previously reported by us in thyroids of rats sacrificed 8 months after a single I^{131} injection) were evident in the parenchyma of all glands. 2. Multiple adenomata, apparently benign in nature, were found in the thyroids of 2 rats. 3. Two types of anaplastic, nonencapsulated neoplasms, one of which was invading adjacent thyroid tissue, were also found in the thyroids of these 2 rats.

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Japanese B Encephalitis in the Rat. (18559)

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It has been reported that the rat can be infected with the virus of Japanese B encephalitis if the virus is injected intratesticularly (1). However, this finding has not been confirmed and it is generally believed that the rat is resistant to infection with Japanese B encephalitis virus(2). The observation that 7- and 8-day-old albino rats are uniformly susceptible to St. Louis encephalitis virus whereas 21-day-old rats fail to develop a clinically apparent infection even though the virus is injected directly into the brain(3,4) suggested that experiments be conducted to determine whether rats of different ages behave toward the related Japanese B encephalitis virus in a similar manner.

The purpose of the present report is to record the data obtained from a study designed to determine the behavior of rats of different ages following injection of Japanese B encephalitis virus.

Materials and methods. Virus and animals. The Nakayama strain of Japanese B encephalitis virus which had been maintained by mouse-brain passage was used.† Mouse brains infected with the virus were ground in a mortar with sand and sufficient of a mixture composed of equal parts of rabbit serum and

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† This strain of virus was obtained through the courtesy of Doctor A. B. Sabin.

TABLE I. Susceptibility of Rats of Different Ages to Japanese B Encephalitis Virus Injected Intracerebrally.

Age, days	No. tested*	Wt		Results		
		Avg, g	Range, g	Mortality Total	Mortality %	Day of death†
7	9	7.6	6.5 to 8.4	9	100	5, 5, 5, 6, 7, 7, 7, 8, 8
7	7	11.5	10.0 to 12.5	7	100	5, 5, 5, 5, 5, 5, 6
12	7	13.4	11.0 to 14.6	7	100	5, 5, 5, 5, 6, 8, 8
12	8	12.9	11.7 to 13.7	8	100	4, 4, 5, 5, 5, 5, 7, 7
12	10	14.0	12.5 to 15.0	1	10	5, S, S, S, S, S, S, S, S, S
21	6	25.9	20.0 to 31.5	0	0	S, S, S, S, S, S
21	6	22.1	17.8 to 24.4	0	0	S, S, S, S, S, S
27	15	36.9	30.0 to 39.1	0	0	S, S, S, S, S, S, S, S, S, S S, S, S, S, S

* At least 2 litters in each age group.
† Numeral indicates day of death. S indicates survival and no evidence of infection.

saline to make a 10% suspension of the infected mouse brains. The suspension was centrifuged at about 2000 R.P.M. for 5 minutes to sediment gross particles. The supernatant liquid was then removed and titrated intracerebrally in 3- to 4-week-old Swiss mice using a mixture of 10% rabbit serum in saline as a diluent. Immediately after the supernatant liquid was removed from the sediment it was distributed in 2 cc amounts in 5 cc glass vials. The vials were sealed and the virus therein was then frozen in a mixture of dry-ice and alcohol. The frozen virus was stored in a dry-ice cabinet. As virus was needed one or more vials were thawed in a water bath at 37°C just prior to the time it was to be used. A single preparation of virus was used in the studies. The mouse intracerebral LD_{50} titer(5) of the freshly prepared virus suspension was $1 \times 10^{-8.8}$. The Fischer strain of albino rats was used.‡ The rats used in the experiments were raised in our animal quarters and an accurate record of their ages was available. All of the rats which were younger than 21 days of age when tested were allowed to remain with the mother. The rats were weighed just prior to inoculation and the average weight and the weight range determined. The inoculated animals were observed daily for 21 days.

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‡ The rats used in the study were kindly supplied by Dr. W. F. Dunning.

Experimental. Behavior of rats of different ages following intracerebral injection of the virus. Rats varying in age from 7 to 27 days were injected intracerebrally with 0.03 cc of the 10% suspension of virus. The results appear in Table I. Of the 16 animals tested when they were 7 days of age all developed a fatal encephalitis. The first signs of infection in these young animals appeared 4 days after the virus was injected. At this time there were no symptoms which would indicate that there was involvement of the central nervous system. However, the animals appeared weak and slightly emaciated. Nine of the 16 animals were found dead on the morning of the 5th day. In those animals which survived longer than 5 days, tremors of the head, ataxia and prostration were observed before death occurred. It is interesting to note that a rat weighing 12.5 g was found to be as susceptible as an animal weighing only 6.5 g. Twenty-five rats were injected intracerebrally with the virus when they were 12 days old. These 25 rats represented 3 different litters. In 16 of the 25 animals a clinically apparent infection occurred and in all 16 the infection had a fatal termination. It is interesting to note that in 2 of the litters tested the mortality was 100%, whereas, in the 3rd litter only 1 of 10 rats injected died of the infection. The course of the disease in rats of this age in which a clinically apparent infection manifests itself, followed by death from the infection, is essentially the same as that described for 7-day-old rats. It

TABLE II. Susceptibility of Young Rats to Japanese B Encephalitis Virus Instilled Into the Nose.

Age, days	No. tested	Wt		Results		
				Mortality		
		Avg, g	Range, g	Total	%	Day of death
7	7	10.7	10.2 to 10.9	7	100	6, 6, 7, 7, 7, 7, 7
12	4	13.3	9.6 to 14.2	4	100	4, 7, 12, 13
12	6	15.1	14.4 to 15.6	4	67	11, 11, 12, 13, S,* S*

* On the 11th day after the virus was instilled into the nose, these 2 animals were weak, emaciated and walked with a staggering gait, but on the 18th day were completely recovered.

should be noted, however, that 2 animals in the 12-day-old group died 4 days after inoculation, whereas, none of the 7-day-old rats died before the 5th day. It will be noted (Table I) that the weights of the 12-day-old rats tested varied from 11.0 to 15.0 g. It would not appear, however, that weight alone had any influence on the susceptibility since there was not a great difference in the average weights of the 3 litters. However, in 2 litters the mortality was 100% and only 10% in the 3rd. The 12 rats which were injected when they were 21 days of age never showed any clinical evidence of infection. The same was true of the 15 rats which were inoculated when they were 27 days of age.

From the data presented in Table I, it is evident that Japanese B encephalitis virus is able to initiate a lethal infection in the rat providing young enough animals are tested. At 7 days of age rats are uniformly susceptible to intracerebral injection of the virus. However, at 12 days of age, while some rats can be infected, others fail to develop clinical signs of infection. Rats which have attained the age of 21 days do not develop an apparent infection even though the virus is injected by the intracerebral route.

Behavior of 7- and 12-day-old rats following nasal instillation of the virus. In this experiment 0.03 cc of the 10% suspension of virus was instilled into the nose of 7- and 12-day-old rats. The results appear in Table II. It will be seen that the virus is highly infectious for these animals when inoculated by this route. All of the 7-day-old rats developed symptoms of encephalitis and the infection terminated fatally in every case. The symptoms observed were the same as those described for the 7-day-old rats which had received the virus by intracerebral injection. Of the ten 12-day-old rats tested 8 developed a fatal encephalitis. In the two 12-day-old rats which survived, the virus apparently invaded the brain and caused encephalitis from which the animals recovered. When these 2 animals were observed on the 11th day after the virus had been instilled into the nose, they appeared weak and emaciated and walked with a staggering gait. Their condition improved gradually on the ensuing days and by the 18th day after inoculation they had recovered. Generally speaking, death occurred earlier in the 7-day-old rats following nasal instillation of the virus than in the 12-day-old group. All of the 7-day-old animals were dead by the 7th day, whereas, in the 12-day-old rats death occurred as late as 13 days after the inoculation of the virus although 1 animal in this age group succumbed on the 4th day. Reference to Table I reveals that only 16 of 25 (64%) rats which were 12 days old when injected intracerebrally with the virus died of the infection. On the other hand, 8 of 10 (80%) of the 12-day-old animals which had the virus instilled into the nose succumbed. This might be interpreted to mean that the 12-day-old animals are more susceptible to the virus when it is instilled into the nose than after intracerebral injection. The probable explanation, however, is that at or near 12 days of age rats begin to become resistant to the virus and the number which are susceptible may vary from litter to litter.

From the data so far presented it is evident that 7-day-old rats are highly susceptible to Japanese B encephalitis virus and that while some 12-day-old animals develop a fatal infection animals in this age group are not

TABLE III. Susceptibility of Young Rats to Japanese B Encephalitis Virus Injected Intraper.

Age, days	No. tested	Wt		Results		
		Avg, g	Range, g	Mortality Total	%	Day of death
7	3	11.9	11.4 to 12.2	3	100	6, 7, 9
8	8	10.2	7.9 to 11.1	8	100	5, 5, 6, 8, 8, 8, 8, 9
8	8	8.6	—	8	100	5, 7, 9, 9, 9, 9, 9, 9
12	9	13.1	11.7 to 14.5	0	0	S, S, S, S, S, S, S, S, S
12	10	12.1	11.2 to 13.0	3	30	5, 12, 12, S, S, S, S, S, S, S

uniformly susceptible. However, rats which are 21 and 27 days of age do not develop a clinically apparent infection.

Behavior of 7-, 8- and 12-day-old rats following intraperitoneal injection of the virus. In this experiment 7- and 8-day-old rats were injected intraperitoneally with 0.1 cc of the 10% suspension of virus and 12-day-old rats were injected by the same route with 0.2 cc of the same virus suspension. The results appear in Table III. The 7- and 8-day-old animals were very susceptible to the virus inoculated by this route since all of the animals tested developed encephalitis and died of the disease. It is interesting to note that death occurred in the 7- and 8-day-old rats which had received the virus intraperitoneally as early as in the 7-day-old rats which had been injected intracerebrally (Table I). This would suggest that the virus, following intraperitoneal inoculation into these young animals, spreads rapidly to the brain to initiate a fatal infection. On the other hand, a high degree of resistance was observed in the 12-day-old rats which were injected intraperitoneally with 0.2 cc of the 10% suspension of virus since in only 3 of the 19 animals in this age group did the virus produce a fatal infection.

Discussion. From the data presented it is apparent that young rats are susceptible to infection with Japanese B encephalitis virus following either intracerebral, intranasal or intraperitoneal injection of the virus. However, as rats grow and develop they become increasingly resistant so that when they attain the age of 21 days they are no longer susceptible to the virus even though it is injected directly into the brain. Duffy and Sabin have reported a similar type of resistance to St. Louis encephalitis virus in the rat. Seven- and 8-day-old rats were found to be susceptible to the virus when it was inoculated by either the intracerebral or intranasal route. On the other hand, rats 21 days old did not develop a clinically apparent infection even though the virus was injected into the brain(3,4).

It would appear that the age-susceptibility pattern of the albino rat to both Japanese B encephalitis virus and St. Louis encephalitis virus is essentially the same since 7- and 8-day-old rats are uniformly susceptible and a high degree of susceptibility is seen in rats 12 days old. At 21 days of age, however, the rat appears to be resistant to both viruses in that rats of this age do not develop a clinically apparent infection, even though the virus is inoculated by the intracerebral route.

Summary. 1. A fatal encephalitis develops in 7-day-old albino rats following nasal instillation or intracerebral injection of Japanese B encephalitis virus. 2. The virus produces a uniformly fatal infection in 7- and 8-day-old rats when injected intraperitoneally. 3. While some 12-day-old rats are susceptible to the virus when inoculated either by the intracerebral, intraperitoneal or intranasal route they are more resistant than 7- and 8-day-old rats. 4. Rats 21 and 27 days of age appear to be resistant to the virus since when it is injected intracerebrally into animals of this age it does not induce a clinically apparent infection.

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Inapparent Infections with Influenza Viruses.* (18560)

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Little information is available concerning the problem of inapparent laboratory infections due to influenza viruses. The ability of this group of viruses to stimulate the production of antibodies has been recognized and extensively studied for many years. This report concerns itself with the development of influenzal antibodies in a group of individuals exposed to several strains of influenza virus for a period of 3-4 months.

Materials and methods. The influenza viruses employed in this study were the strains PR 8, Lee and FM-1. The 3 strains were adapted laboratory preparations in either allantoic fluid or as mouse lung suspensions. The strains were stored in the frozen state, or at 4°C, during the course of the experiment. Sera were obtained from 65 students, composing the class of 1953 (State University of New York Medical School, Syracuse, N. Y.), on 3 different occasions. The first serum specimen was obtained approximately 2 months before the second specimen, the third blood sample was obtained approximately 5 weeks after the second. Eight of the 65 students were engaged in a problem involving the use of the 3 strains of virus (test group). The start of this problem coincided with the first blood specimen. The second blood sample was obtained several days before *all* the students became engaged in the inoculation, harvesting and titration (hemagglutination-inhibition tests) of these influenza strains in chick embryos and mice. These experiments were completed in a period of 3 days, for approximately 2 hours each day. The last blood sample was taken after a period involving no known virus contact. All sera were stored at −20°C until tested. Inactivation of the sera was accomplished by heating at 60°C for 3 minutes. Titrations of the three serum samples were made simultaneously by the hemagglutination-inhibition test.

Experimental. The results indicate that significant antibody increases developed in the group of students in contact with the viruses for the continued period of time. Six of the 8 students (test group) showed evidence of 4-fold or better increases in antibodies against the 3 strains. These results and representative titers of the control group are tabulated in Table I. None of these 8 students indicated at any time during this period any clinical syndrome resembling influenza. As indicated, there was no demonstration of antibody increases in the control group.

One observation, made on the control group, was the uniformly higher titers obtained against the Lee strain. In the majority of specimens examined, the titer for this virus was consistently higher than those obtained for the PR 8 and FM-1 strains.

Discussion. Subclinical or inapparent infections with influenza viruses have been reported: (a) during epidemics, (b) between epidemic periods, and (c) during epidemics due to influenza viruses of another type(1-3). However, little data are available regarding inapparent laboratory infections. The data reported here indicate that inapparent infections due to influenza viruses may occur. The data also suggest that a continued exposure to the virus is required.

The failure to develop clinical infection apparently is analogous to the situation described by Henle and coworkers(4). This group demonstrated that the inhalation of influenza viruses did not cause a febrile reaction. To this may be added the possibility that

* Aided by a grant from the Hendricks Research Fund.

1. Burnet, F. M., Cade, J. F. J., and Lush, D., *M. J. Australia,* 1940, v1, 397.

2. Salk, J. E., Menke, W. J., Jr., and Francis, T., Jr., *Am. J. Hyg.,* 1945, v42, 57.

3. Finland, M., Barnes, M. W., Meads, M, and Ory, E. M., *J. Lab. and Clin. Med.,* 1948, v33, 15.

4. Henle, W., Henle, G., Stokes, J., Jr., and Maris, E. P., *J. Immunol.,* 1946, v52, 145.

TABLE I. A Comparison of the Antibody Levels in Sera from Individuals Exposed to Influenza Virus for Several Months and a Few Days (Control Group).

Test group				Control group*			
Serum No.	PR 8	Lee	FM-1	Serum No.	PR 8	Lee	FM-1
			Final titer				
1A	160	640	40	9A	80	320	320
B	1280	5120	320	B	80	320	320
C	1280	2560	320	C	80	320	320
2A	80	160	160	10A	160	640	160
B	640	2560	1280	B	160	320	80
C	640	2560	1280	C	320	320	160
3A	160	160	160	11A	320	640	640
B	1280	640	640	B	320	640	640
C	1280	640	640	C	320	640	640
4A	320	160	160	12A	80	640	160
B	10240	1280	640	B	80	640	160
C	10240	5120	640	C	160	640	160
5A	640	640	640	13A	80	80	80
B	1280	1280	1280	B	40	80	80
C	2560	1280	1280	C	40	80	80
6A	320	160	2560	14A	320	640	160
B	2560	640	2560	B	160	640	80
C	5120	640	2560	C	160	320	160
7A	160	320	160	15A	80	320	80
B	2560	5120	5120	B	80	320	80
C	2560	5120	5120	C	80	320	80
8A	80	320	160	16A	320	1280	640
B	2560	2560	5120	B	320	1280	320
C	2560	2560	2560	C	640	1280	640

* Eight of 57 students.

these strains are unable to cause a typical clinical picture in man after several years of animal or chick embryo passage.

The reason for the high titers against the Lee strain is obscure. This observation, however, has been made previously(3,5). There are several possible explanations: (1) sporadic inapparent infections with Influenza B occurring in the population previous to this study, and (2) residual titers from a previous infection with this virus.

Summary. Data are presented which suggests that inapparent infections with influenza viruses may occur after continued exposure for relatively long periods of time.

Our appreciation to Miss Phyllis Miner for her technical assistance and to the Class of 1953 for their cooperation.

5. Lazarus, A. S., and Westfall, R. E., *J. Immunol.*, 1948, v59, 159.

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Distribution of Mumps Virus in the Experimentally Infected Monkey.* (18561)

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With the recent development of technics for the identification of mumps virus by serological tests(1), and for its propagation

* This work was supported by grants from the Helen Hay Whitney Foundation and the National Foundation for Infantile Paralysis.

1. Enders, J. F., and Cohen, S., PROC. SOC. EXP. BIOL. AND MED., 1942, v50, 180.

in the embryonated egg(2,3), considerable information has been obtained on the distribution of this agent in the human host in the course of natural infection. Mumps virus has been isolated from saliva(4), spinal fluid(5), blood(6), testis(7), parotid gland (8), and either pancreas or ovary(8). It is apparent that this agent can exhibit on occasion pantropic characteristics in the course of natural infections in man. Although Johnson and Goodpasture showed as early as 1934(9) that the monkey could be experimentally infected via Stensen's duct, relatively little is known about the distribution of mumps virus in this host following this procedure. The agent has been isolated from the parotid gland(2,3) which as yet is the only organ in which complement fixing antigen has been demonstrated. More recently Coons, *et al.*(10) have microscopically localized mumps virus antigen in this organ by means of homologous antibody labelled with fluorescein. The present paper deals with a more extensive search for mumps virus in the various organs of one experimentally infected monkey. The individual tissues were first examined for the presence of mumps virus antigen by means of fluorescent microscopy and specific antibody labelled with fluorescein. The same tissues were then tested for the presence of infectious virus by the classical method of inoculation of the embryonated egg.

Materials and methods. The Enders strain of mumps virus was employed. A normal young adult male rhesus monkey (Macacca mulatta) was infected by the bilateral injection of approximately 0.5 ml-1 ml of a 1:40 suspension of infected parotid gland into Stensen's duct. At the end of 96 hours (before any glandular swelling or other gross evidence of infection had developed) the animal was sacrificed and autopsied. Individual tissues were frozen and stored in CO_2 cabinet until the time of testing.

Use of fluorescein-labelled antibody in the search for mumps virus. Convalescent anti-mumps monkey serum was concentrated, conjugated with fluorescein isocyanate and purified as previously described(10,11). Small portions of it were absorbed with mouse liver powder and used to localize mumps virus in tissue sections. These were prepared from quick-frozen tissue in a cryostat by the method of Linderstrøm-Lang and Mogensen as modified in this laboratory(12). It was found that exposure of such frozen sections to fluorescein-antibody solutions often resulted in the formation of a brightly fluorescent scattered precipitate unrelated to the presence of mumps virus; this was especially troublesome in the case of central nervous system tissues. Placing the sections attached to slides in acetone at room temperature for 15 minutes, and then allowing them to dry for 30 minutes at 37° abolished this non-specific precipitation. We have no ready explanation for this phenomenon. The sections, after treatment with fluorescein anti-mumps conjugate for 30 minutes, were washed, mounted, and examined under the fluorescence microscope. Control of specific staining was accomplished as follows. Companion sections were treated with normal monkey serum and anti-mumps monkey serum respectively (both unlabelled), followed by exposure of both sections to labelled anti-mumps serum. Fluorescence due to mumps virus was absent when inhibited or "blocked" under appropriate conditions by specific unlabelled antibody. This technic has been described in detail elsewhere(10).

2. Habel, K., *Public Health Rep.*, 1945, v60, 201.

3. Levens, J. H., and Enders, J. F., *Science*, 1945, v102, 117.

4. Leymaster, G. R., and Ward, T. G., PROC. SOC. EXP. BIOL. AND MED., 1947, v65, 346.

5. Henle, G., and McDougell, C. L., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 209.

6. Kilham, L., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 99.

7. Friedewald, W. F., *Am. J. Med.*, 1949, v7, 257.

8. Weller, T. H., and Craig, J. M., *Am. J. Path.*, 1949, v15, 1105.

9. Johnson, C. D., and Goodpasture, E. W., *J. Exp. Med.*, 1934, v59, 1.

10. Coons, A. H., Snyder, J. C., Cheever, F. S., and Murray, E. S., *J. Exp. Med.*, 1950, v91, 31.

11. Coons, A. H., and Kaplan, M. H., *J. Exp. Med.*, 1950, v91, 1.

12. Coons, A. H., Leduc, E. H., and Kaplan, M. H., *J. Exp. Med.*, 1951, v93, 173.

Isolation of virus. After frozen sections had been prepared for histochemical studies the remainder of the tissue fragment was ground with alundum in isotonic phosphate buffer solution to make a 10% suspension, and after light centrifugation, penicillin was added to the supernatant to give a final concentration of 500 units per ml. Embryonated eggs 7 or 8 days old were inoculated amniotically, and one week later the amniotic fluids were harvested individually and tested for the presence of hemagglutinins. Specific serological identification was carried out by the hemagglutin-inhibition technic as outlined by Robbins *et al.*(13). In the absence of hemagglutination two blind passages employing pooled amniotic fluids (and occasionally amniotic membranes as well) were usually carried out before the original material was considered to be negative.

Results. I *Identification of mumps virus antigen by homologous antiserum labelled with fluorescein.* The positive findings were limited to the parotid glands, the spinal cord, the medulla, and possibly the cerebrum and cerebellum. In both parotid glands specific antigen was demonstrated chiefly in the cytoplasm of the acinar cells. These findings have been described elsewhere(10). In the spinal cord the antigen (presumably for the most part active mumps virus) was found scattered in the white matter in small rather amorphous irregular globules. No clear cell outlines were visible, and whether it was intra or extracellular could not be determined. It was irregularly distributed. In the cervical cord a moderate amount of staining was found just lateral to one dorsal horn, with smaller scattered amounts in the opposite ventral and lateral fibertracts. In the lumbar enlargement rather more staining was visible, this time in the ventral white matter lying between the emerging motor fibers from ventral horn on each side. The overlying meninges showed no antigen.

In the medulla a trace of similar amorphous material was found just beneath the floor of the 4th ventricle, which seemed to be in the cytoplasm of cells.

The examination of the cortex and cerebellum was unsatisfactory because it was undertaken first. The scattered non-specific precipitate described above (Methods) made definite findings impossible. By the time it was found that treatment of the sections with acetone mitigated this difficulty, the supply of tissue was exhausted.

The meninges covering the cortex were repeatedly examined, without finding specific fluorescence. Kidney, liver, spleen, adrenal, lung, heart, muscle, testis, one lymph node, fat tissue, stomach, small bowel, large bowel and pancreas showed no staining specific for mumps antigen.

II *Virus isolation.* Mumps virus was isolated from 7 of the 22 tissues examined. Positive results were obtained in the case of: left parotid gland, right parotid gland, meninges, cortex, cerebellum, cervical cord and lumbar cord. No virus was isolated from the following organs: liver, adrenal gland, kidney, lung, spleen, lymph node, heart, skeletal muscle, pancreas, fat, stomach, small bowel, large bowel, testis and medulla. In all 7 positive cases hemagglutinins were detected in the amniotic fluid of the first egg passage: in 2 of the 7 the titers were high enough to permit serological identification without further passage. The virus was identified by means of the hemagglutation-inhibition test utilizing first egg passage material in the case of both parotid glands; second egg passage material in the case of cortex, meninges, cerebellum and lumbar cord; and third egg passage material in the case of cervical cord.

As regards the organs from which virus could not be isolated, three egg passages were carried out before the material was discarded, with the exception of the lung which was passed but once. These results are summarized in Table I.

Discussion. Of considerable interest was the demonstration of mumps virus in widely scattered areas of the CNS only 4 days after inoculation of the monkey by the injection of Stensen's ducts. During these 4 days no signs pointing to the involvement of the CNS were observed. Presumably the route of infection was the hematogenous one; unfor-

13. Robbins, F. C., Kilham, L., Levens, J. H., and Enders, J. F., *J. Immunol.*, 1949, v61, 235.

TABLE I. Detection of Mumps Virus Antigen and Isolation of Mumps Virus.

Tissue	Detection of antigen	Isolation of virus
Left parotid gland	+	+
Right " "	+	+
Kidney	—	—
Liver	—	—
Spleen	—	—
Adrenal	—	—
Lung	—	—
Heart	—	—
Skeletal muscle	—	—
Pancreas	—	—
Lymph node	—	—
Fat	—	—
Stomach	—	—
Small bowel	—	—
Large bowel	—	—
Testis	—	—
Meninges	—	+
Cortex	?	+
Cerebellum	?	+
Medulla	+	—
Cervical cord	+	+
Lumbar cord	+	+

tunately the blood was not examined for the presence of virus. Since, however, the virus could not be detected in the other organs examined (with the exception of the parotid glands) it seems unlikely that it was present in the blood in high concentration at the time the animal was sacrificed. Had the experiment been permitted to run longer than 96 hours the distribution of virus might have been different. The results are compatible with the clinical observation that the CNS of man may be invaded by the virus during the course of natural infections.

The data presented in the present paper furnish additional evidence as to the value of fluorescein labelled antibody as a histochemical tool. Fair agreement was produced by the two methods—virus isolation and demonstration of viral antigen—employed to investigate the distribution of mumps virus in the experimentally infected monkey. In 14 of the 22 tissues examined the presence of virus could not be demonstrated by either method. In 4 tissues both methods yielded positive results. Virus was isolated from 2 tissues (cortex and cerebellum) in which the application of labelled antibody yielded questionable results due to technical difficulties. One tissue (medulla) gave a positive reaction with labelled antibody, but no virus was isolated from it. Conversely, no mumps antigen could be detected in the meninges, although the virus was itself isolated from both samples of this tissue that were tested. Thus, of 22 trials the methods were in agreement 18 times. In two instances they yielded divergent results, while in the case of 2 tissues, the results could not be compared because of technical difficulties.

Summary. The distribution of mumps virus in one experimentally infected monkey 96 hours after inoculation has been investigated by simultaneous labelled antibody and virus isolation studies. The results furnished by the two methods were in fair agreement. Virus was demonstrated in CNS (brain and spinal cord) as well as in both parotid glands, although the animal had shown no neurological signs. No virus was demonstrated in the other organs examined.

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Lack of Effect of Folic Acid on Scurvy in Guinea Pigs. (18562)

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The frequent association of scurvy with megaloblastic anemia in infants was pointed out by Zuelzer(1) in his initial description of this hematologic disorder. Scurvy was then considered only as a manifestation of the poor nutritional status of the patient and other-

* Postdoctorate Research Fellow, National Institute of Health, U. S. Public Health Service.

1. Zuelzer, W. W., and Odgen, F. N., *Am. J. Dis. Child.*, 1946, v71, 211.

wise unrelated to megaloblastic anemia. The possibility of a closer relationship between vitamin C and folic acid has been suggested by reports of folic acid deficiency in rats which responded to the administration of ascorbic acid(2,3). More recently May(4) has stated his belief that chronic deficiency of ascorbic acid leads to a deficiency of folic acid or related compounds which results in a megaloblastic pattern in the marrow.

In an attempt to isolate the effect of folic acid on megaloblastic anemia and scurvy, a 6-month-old male infant in this hospital, afflicted with both diseases, was placed on a low vit C diet and given therapeutic amounts of folic acid. The hematologic response was excellent and corresponded to that described by Zuelzer. Serial roentgenograms of the long bones suggested that the scorbutic process was also ameliorated during the administration of folic acid in the presence of a low and presumably inadequate vit. C intake. These observations led us to undertake studies to determine the effect, if any, of folic acid on vit. C deprivation in the guinea pig.

Male guinea pigs weighing approximately 150 g were fed, *ad libitum,* a scorbutogenic diet(5). Normal growing guinea pigs on this diet develop signs of scurvy within 18 to 25 days and die shortly after. Ten animals were given 1.5 mg of folic acid† twice daily by subcutaneous injection. Nine animals were given an equivalent volume of physiological saline solution subcutaneously twice daily. Two animals receiving no treatment were given ascorbic acid beginning on the twenty-first day of the diet. The animals were weighed daily and at death were autopsied. As this was initially considered a pilot experiment, only death rates were compared in the 2 groups; phosphatase determinations as described by Gould and Shwachman(5) were not made. The cumulative death rates were practically identical, all the animals in both groups having died by the thirty-second day. The experiment was therefore repeated using 250 g male guinea pigs and 3 mg of folic acid† twice daily; the control animals were given an equivalent volume of physiologic saline solution. Cumulative death rates were the same as in the initial experiment. Six untreated animals on the scorbutogenic diet died between the nineteenth and twenty-ninth days. The combined cumulative mortality rates for the 2 experiments are shown in Fig. 1. Of the 2 animals given ascorbic acid beginning on the twenty-first day of the diet in the first experiment, one died after 6 days. The other recovered, and was sacrificed on the thirty-seventh day of the diet. Histologic study of sections from the region of the knees in representative animals in each group showed characteristic changes of active scurvy in all except the animals treated with ascorbic acid; these showed healing or healed scurvy.

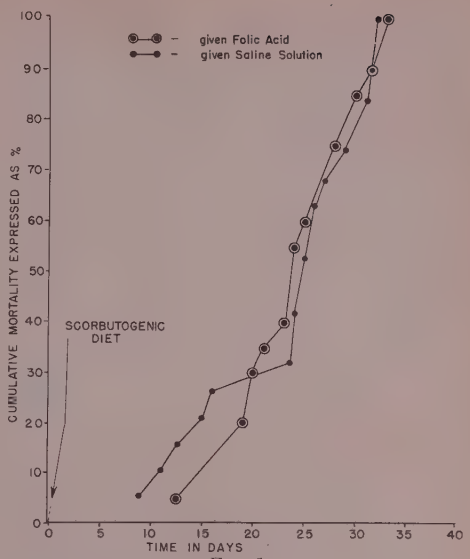

FIG. 1.

Cumulative mortality rates of guinea pigs on scorbutogenic diet.

Discussion. The evolution of scurvy and the death rates are those to be expected in

2. Johnson, B. C., and Dana, A. S., *Science,* 1948, v108, 210.

3. Woodruff, C. W., and Darby, W. J., *J. Biol. Chem.,* 1948, v172, 851.

4. May, C. D., Nelson, E. N., Lowe, C. U, and Salmon, R. J., *Am. J. Dis. Child.,* 1950, v80, 191.

† Folic acid was administered as Folvite supplied through the courtesy of Lederle Laboratories.

5. Gould, B. S., and Shwachman, H., *J. Biol. Chem.,* 1943, v151, 439.

guinea pigs on the scorbutogenic diet if antiscorbutic substances are withheld. Folic acid apparently did not affect the animals favorably or adversely. An adverse effect might have been expected if larger doses of folic acid were given, since the guinea pig is known to be more susceptible to toxic effects of folic acid than other animals(6). On a weight basis, the dosage given was greater than that used therapeutically in humans. The development of scurvy in monkeys receiving folic acid has been observed by May (4). The roentgen observations of amelioration were therefore puzzling. Later, a careful review of records revealed that a single dose of 200 mg of ascorbic acid, administered intravenously as part of a tolerance test performed to establish the diagnosis of scurvy, could have been responsible for the regression of the skeletal lesions noted roentgenographically.

Conclusions. Under conditions of vit. C deprivation, folic acid has no apparent antiscorbutic effect in guinea pigs.

6. Harned, B. K., Cunningham, R. W., Smith, H. D., and Clark, M. C., *Ann. New York Acad. Sc.*, 1946, v8, 289.

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Thioglycollate Inactivation of Posterior Pituitary Antidiuretic Principle as Determined in the Rat.* (18563)

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Recently, Ralli and her collaborators(1) suggested that "the antidiuretic activity of commercial Pitressin can be fractionated into at least two substances, one of which is active in the rat but not in the dog." This suggestion was based upon the observation that the antidiuretic action of Pitressin could be abolished by treatment with 0.01 M sodium thioglycollate(2) as determined by intravenous assay in dogs but not by similar treatment of Pitressin when assayed by intraperitoneal injection in rats. On the other hand, the chloruretic action of large doses of Pitressin treated with thioglycollate was reduced 45% in comparison with untreated Pitressin. (Whether the chloruretic action was abolished cannot be determined from the published experiments which lacked control groups receiving no Pitressin.) Ralli and her colleagues also concluded that in the dose range of 10-100 milli-units the chloruretic effect of Pitressin in rats was related to its antidiuretic action.

In their study of the antidiuretic action of thioglycollate-treated Pitressin, Ralli *et al.*(1) reported control experiments only with Pitressin containing a similar concentration of acetate. Sodium thioglycollate (1 ml of 0.01 or 0.05 M solution) has no antidiuretic effect after intravenous injection into the dog(2). However, its action on water diuresis in the rat after either intraperitoneal or intravenous injection has never been reported. In the experiments about to be described it was found that solutions of sodium thioglycollate have an "antidiuretic" action after intraperitoneal or subcutaneous injection into rats but not after intravenous injection.

Material and methods. Adult rats of either sex of the Long-Evans strain were used. In all the experiments a special lot of Pitressin†

* This investigation was in part supported by grants from the Playtex Park Research Institute and E. R. Squibb and Sons.

1. Ralli, E. P., Raisz, L. G., Leslie, L. H., Dumm, M. E., and Laken, B., *Am. J. Physiol.*, 1950, v163, 141.

2. Ames, R. G., Moore, D. H., and van Dyke, H. B., *Endocrinology*, 1950, v46, 215.

† The Pitressin was kindly furnished by Dr. A. C. Bratton of Parke, Davis and Co.

containing 80 units of antidiuretic (pressor) activity and 3.8 units of oxytocic activity per mg was used. When injections were made intraperitoneally or subcutaneously urine was collected from groups of 4 rats in metabolism cages. Each rat received by stomach tube at the start of the experiment 5 ml of water per 100 g weight. The volume of urine excreted was recorded every 15 minutes. The method of Jeffers, Livezy and Austin(3) was employed for the intravenous assays. The volume of each dose usually was 0.5 ml or less. The volume of urine excreted was determined every 5 minutes.

Experimental results. One of the 5 experiments performed in groups of rats receiving solutions either intraperitoneally or subcutaneously is summarized in Table I and is typical of the results obtained. The "antidiuretic" effect of sodium thioglycollate as a 0.01 M solution intraperitoneally was just as great as 25 milli-units of Pitressin with or without thioglycollate. The larger intraperitoneal dose of Pitressin, 100 milli-units, interfered with diuresis more than thioglycollate alone or thioglycollate and 100 milli-units of Pitressin. It must be emphasized that an effort to secure results of considerable accuracy by using a number of groups at each dose was not believed to be necessary. The

TABLE I. Action of Pitressin or Sodium Thioglycollate Solution or a Mixture of Both on Water Diuresis in the Rat.

Group*of 4 rats	Route of injection	Min. required for excretion of 50% of dose of water	
Control†	Intraper.	80	
.01 M NaTG‡	"	210	
.01 M NATG cont. 25 m.u. Pitressin	"	210	
25 m.u. Pitressin	"	195	
.01 M NaTG cont. 100 m.u. Pitressin	"	150	
100 m.u. Pitressin	"	255	
.01 M NaTG	Subcut.	>270	35%
.01 M NaTG cont. 100 m.u. Pitressin	"	>270	45%
100 m.u. Pitressin	"	>270	12%

* All doses 1 ml.
† Received isotonic saline sol.
‡ NaTG = sodium thioglycollate.

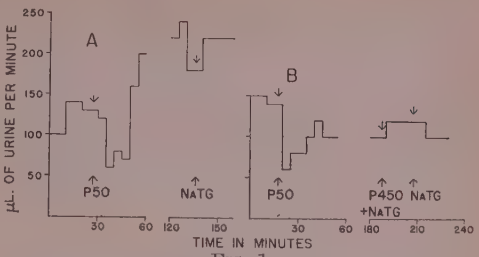

FIG. 1.

Effect of intravenous injections upon urinary excretion in adult rats. P50: 50 μu. of Pitressin in .5 ml of saline (both exp.). P450 + NaTG: 450 μu. in .5 ml of saline containing .01 M sodium thioglycollate (exp. B). NaTG: 1 ml of .01 M sodium thioglycollate (both exp.).

figures given in the experiment of Table I are consistent with the results of other experiments and explain the antidiuretic effect obtained in rats by Ralli and her co-workers when they injected mixtures of Pitressin and sodium thioglycollate intraperitoneally: the Pitressin could have been inactivated and the "antidiuretic" action of the mixture could have been attributed to the thioglycollate.

The delay in diuresis after the subcutaneous injection of the solutions as shown in Table I is, as would be expected, even greater than after intraperitoneal injection. The percentages in parentheses indicate the fractions of administered water excreted as urine after 270 minutes.

One ml of 0.01 M sodium thioglycollate administered to adult rats either subcutaneously or intraperitoneally is not innocuous. Deaths occurred (1 or 2 of the 4 in the group) 12 to 24 hours after injection in every group of Table I receiving thioglycollate except the one to which the Pitressin-thioglycollate mixture was administered subcutaneously.

In the intravenous assays in rats, several injections can be made into each animal. Typical results from 2 of the experiments are shown in Fig. 1. Although the volume of doses usually was 0.5 ml or less, even 1 ml of 0.01 M sodium thioglycollate intravenously caused no interference with diuresis during the period of observation. In experiment B, an inactivation of Pitressin by thioglycollate similar to that found by intravenous assays in the dog, was demonstrated.

Summary and conclusions. After intraperi-

3. Jeffers, W. A., Livezy, M. M., and Austin, J. H., PROC. SOC. EXP. BIOL. AND MED., 1942, v50, 184.

toneal or subcutaneous injection, an aqueous solution of sodium thioglycollate markedly reduces water diuresis in rats and cannot be used by either route to demonstrate unequivocally whether posterior pituitary antidiuretic principle has been inactivated. By the intravenous route in rats, thioglycollate does not interfere with diuresis and can be shown, as in the dog, to abolish the antidiuretic action of Pitressin. Therefore, there is no controlled experimental evidence that Pitressin contains more than one antidiuretic principle.

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Stimulation of Cell-Free Gastric Mucus by the Topical Application of Acetylcholine.* (18564)

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This laboratory has been concerned for several years with the systematic study of the characteristics of gastric mucus(1-4). In the course of these investigations the secretion of gastric mucus has been stimulated by the topical application of a wide variety of watery solutions and emulsions, of which eugenol (4-allyl,2-methoxyphenol), the chief constituent of clove oil, was found to be the most effective stimulus for mucus secretion. The mucus obtained by means of these topical stimuli usually, but not always, contained considerable numbers of intact columnar epithelial cells. Thus of 42 smears of specimens secreted in response to the topical application of 5% eugenol to canine fundic pouches, only 7 (17%) had few or no columnar cells [*i.e.*, at most a small number per low power microscopic field], while 35 (83%) had many columnar cells. These cells occurred not only in mucus stimulated by such irritating substances but also in that secreted "spontaneously" in very small amounts by the resting pouch. Thus, of 100 specimens secreted without topical stimulation, 70 (70%) had few columnar cells, while only 30 (30%) contained many. From these studies (3) it was concluded that pure mucus, as it is secreted, is of variable consistency but free of suspended desquamated columnar cells; although desquamation can occur concurrently with such secretion, the exfoliation of cells is not an integral part of the secretory process.

It therefore became necessary to develop a method for stimulating the output of cell-free gastric mucus in considerable quantity, in order both to confirm these inferences and to have a cell-free mucus suitable for further physical and chemical studies. Stavraky and Morton(5) had shown, in the dog, that intra-arterial injection of acetylcholine into the gastrosplenic artery (which supplies the corpus of the stomach along the greater curvature) results in the secretion of mucus. These experiments were performed with acute preparations, but they suggested that topical stimulation of the mucosa of canine fundic pouches by acetylcholine might be equally effective and more serviceable as a reproducible method for obtaining mucus in intact unanesthetized animals.

Methods. Four mongrel dogs with Heidenhain gastric pouches were employed in this study. The pouches were prepared with the conventional technic from the greater curva-

* This investigation was carried on with the aid of grants from the National Cancer Institute (U. S. Public Health Service), and the Altman Foundation.

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2. Hollander, F., *Gastroenterology,* 1944, v3, 638.

3. Hollander, F., Stein, J., and Lauber, F. U., *Gastroenterology,* 1946, v6, 576.

4. Hollander, F., and Lauber, F. U., PROC. SOC. EXP. BIOL. AND MED., 1948, v67, 34.

5. Morton, G. M., and Stavraky, G. W., *Gastroenterology,* 1949, v12, 808.

ture area of the body of the stomach. These animals were invariably starved for 18-24 hours before each experiment. They were then suspended according to the technic usually employed in this laboratory for the collection of gastric juice from pouch dogs, and the spontaneous secretion was collected by soft rubber collecting tubes. The collection of unstimulated secretion was continued until its pH was at least 6.0, and only then was the stimulus applied. For such topical stimulation the animal was removed from its sling and the collecting device was replaced by a pezzer (mushroom) catheter—No. 18-22 Fr. The stimulating fluid was then slowly injected into the pouch, the volume being such as to fill the pouch completely without the application of any considerable amount of syringe pressure. This quantity ranged between 30 and 50 cc. The fluid was withdrawn at the end of 15 minutes, and a fresh supply injected for a second 15-minute period. At the completion of these instillations, the dog was returned to the sling, the excess stimulating fluid was drained off, and collection of mucus was begun. The volume secreted was noted at 30-minute intervals and collections were continued until the rate of secretion fell to insignificant values. Besides acetylcholine chloride (ACh) (1 mg/cc), mecholyl chloride, (0.5 mg/cc), and pilocarpine hydrochloride (1 mg/cc) were also used. The solutions were made up freshly each time, just prior to use, in normal saline or distilled water. These doses of the drugs were tolerated without any toxic manifestations. Viscosity measurements were done with the Fenske-Ostwald viscosimeter at 37°C. Measurements of pH were done either with the glass electrode or with indicators.

Results. Collection of mucus. Application of 30 to 50 cc of solution to the mucosa of the Heidenhain pouch for 30 minutes resulted in the secretion of an extremely viscous opalescent jelly-like mucus. This highly tenacious material was first noted in the collecting graduated tube during the second 30-minute interval following stimulation, and it continued to drain for 3 to 5 hours thereafter. Application of mecholyl or pilocarpine solutions for 30 minutes to these fundic pouches yielded similar material.

In 37 such experiments, the volume of mucus obtained during the entire collection period ranged from 1.7 to 12.0 cc (mean = 5.2 cc). The duration of these collections averaged 4.4 hours. The magnitude of the response appeared to be unrelated to this duration, probably because of the slow and irregular drainage of such viscous material. In the dosage employed, ACh gave a slightly greater response than did mecholyl or pilocarpine. Thus in 27 experiments with ACh, the volume ranged from 2.0 to 12.0 cc (mean = 5.8 cc); in 6 experiments with mecholyl it varied from 2.3 to 5.3 (mean = 4.5); and in 4 experiments with pilocarpine the range was 1.7 to 6.3 (mean = 4.0).

Characteristics of cell-free mucus. Grossly, the specimens were jelly-like, extremely viscous, translucent to opalescent, with occasional small particles of coagulated material. Microscopic examination of smears of this material, stained with toluidine blue or the periodic acid-Schiff technic revealed no individual intact columnar cells or groups of cells in the metachromatically staining background of mucin, and only a few scattered nuclei. The "relative" viscosity of this ACh-stimulated mucus freshly secreted ranged from 29 to 260 (mean = 93). Its pH was between 7.0 and 7.5. The material collected after stimulation with pilocarpine or mecholyl possessed essentially the same characteristics.

Discussion. These experiments indicate clearly that the topical application of acetylcholine or related parasympathomimetic drugs to the mucosa of Heidenhain canine pouches results in the secretion of highly viscid opalescent alkaline cell-free mucus. These findings are consistent with the reported results following the intra-arterial injection of ACh, and furnish a simple convenient method for collecting mucus secretion from fundic pouches for the study of its physical and chemical characteristics.

Of primary interest is the finding that this ACh-stimulated mucus is essentially free of cells. This is in marked contrast to the desquamating action of all the topical muci-

gogues previously studied, both in this laboratory and in others. It thus confirms the inference which we made on the basis of previous correlation studies: that visible mucus is secreted free of cells, and that the desquamation of surface columnar cells is not an integral part of the process of mucus secretion(3).

In contrast to the opacity of mucus secreted in response to the previously studied chemical mucigogues, which opacity has been found to be correlated in high degree with its columnar cell content, the mucus stimulated by ACh or related drugs is much clearer, being opalescent with a small number of more opaque fragments suspended in the mucoid mass. These fragments probably represent small particles of coagulated mucin.

In the concentrations used in the present work, ACh compares favorably with eugenol as far as rate of secretion is concerned. Thus in 65 experiments with 5% eugenol, the mean volume secreted was 6.4 cc(4), whereas in those with ACh it was 5.8 cc. But analysis of these rates is complicated by the extreme viscosity of the material secreted, with resulting slowness and irregularity of collection.

Because of its small cellular content and few gross particles, viscosimetry of native mucus proved feasible with the Ostwald technic. In this way, considerable variation in the viscosity of this ACh-stimulated mucus was observed from experiment to experiment. The meaning of such variations must await more detailed study of the chemical composition of specimens obtained by this procedure.

These studies do not furnish evidence on the mechanism of this ACh stimulation of mucus secretion, but experiments to this end are already under way.

Summary. The topical application of acetylcholine or related parasympathomimetic drugs to the mucosa of canine fundic pouches results in the secretion of alkaline opalescent viscid mucus which is free of columnar cells. These studies confirm our previous conclusion, that the exfoliation of gastric surface cells is not an essential part of the process of mucus secretion.

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Plasma Cholesterol Levels of Cholesterol Fed Control and Pyridoxine Deficient Monkeys.* (18565)

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There is little information on the effects of feeding cholesterol to the rhesus monkey. Mild increases of serum cholesterol have been observed in this species by Sperry, Jailer and Engle(1) following the feeding of whole egg or egg yolks as a source of cholesterol. The present paper describes the effect of cholesterol feeding on the plasma cholesterol of pyridoxine deficient and control monkeys and was carried out in connection with our studies on the development of arteriosclerotic lesions in vitamin B6 deficient monkeys(2,3).

Experimental. Studies on two groups of young rhesus monkeys are reported in the present paper. The first included 8 monkeys (7 males and 1 female) weighing 1.85-2.48 kilos which were housed in individual cages. They were fed *ad libitum* the modified M-3 basal diet and the complete supplements described in a previous publication(4) for a period of 39 days. Pyridoxine was then with-

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TABLE I. Plasma-cholesterol Levels in Control and Pyridoxine-deficient Monkeys.

No. and status	Control period: Deficient period before cholesterol, days	Control period: Range, mg %	Control period: Avg, mg %	Cholesterol period: Range, mg %	Cholesterol period: Avg, mg %	Cholesterol period: Avg increase, %	Cholesterol period: Days†	Avg daily food consumption, g
				Group I				
12 D	108	76-131	103	124-487	334	224	145	80¶
13 D	108	79-119	99	129-422	260	163	145	71
14 D	59	75-100	87	165-491	303	248	76§	76
15 D	59	96-103	99	156-541	328	231	204	91
19 C	—	157-193	175	109-296	213	22	204	280
16 D	—	92-208	148‡					
17 D	—	112-237	174‡					
18 C	—	81-188	138‡					
				Group II				
36 D	60	89-192	98	220-533	419	328	77	239
37 D	60	155-252	207	351-765	513	148	77	129
38 C	—	165-219	182	263-429	360	99	77	513
35 D	—	148-239	192‡					

* D or C (deficient or control).

† Avg in Group I include only those values obtained to time of administration of 3.5 mg doses of pyridoxine.

‡ Computed for combined intervals corresponding to control and cholesterol periods of the sterol-fed animals.

§ Sacrificed because of comatose condition resulting from marked pyridoxine deficiency.

‖ No cholesterol.

¶ Approx. value, this monkey wasted considerable amounts of food.

drawn from the diet of 6 animals, and one per cent cholesterol was added to the diet of one control and 4 deficient monkeys at the times indicated in Table I. One control and 2 deficient monkeys were allowed to remain on the basal diet without added cholesterol. The cholesterol was incorporated into the diet by merely intimately mixing the finely powdered sterol with the remaining ingredients of the diet. During the course of the experiment the plasma cholesterol levels were followed at frequent intervals for 65 weeks or longer by the method of Sackett(5). The blood used for the analyses was drawn during the early part of the morning before the day's food ration was placed in the monkeys' cages.

Results. The plasma cholesterol values for 3 of the monkeys, 2 deficient (nos. 12 and 13) and 1 control (no. 19) are shown graphically in Fig. 1. The ranges of the plasma cholesterol together with their average values and the average percentage increases are summarized in Table I.

The curves of the 3 monkeys in Fig. 1 are typical of the results observed in the other similarly treated members of the group and show clearly the course of the plasma cholesterol following the addition of the sterol to the diet. In the course of about 4 weeks after initiation of the cholesterol feeding, the plasma of the deficient monkeys 12 and 13 exhibited marked increases and consistently higher cholesterol levels than no. 19, the corresponding control animal. The average increase in the level of no. 19 was only 22% while during the same period the average increases in the levels of nos. 12 and 13 were 251 and 197%, with maximum values of 487 and 422 mg % respectively. The 2 other cholesterol-fed, deficient animals (nos. 14 and 15) of Group 1 attained average increases of 248 and 231%, and maximum values of 491 and 541 mg % respectively. The greater rise in the plasma cholesterol of the sterol fed deficient monkeys is all the more remarkable when one considers the fact that the average daily food consumption of these monkeys was only approximately ⅓ to ¼ that of the control animal, and hence

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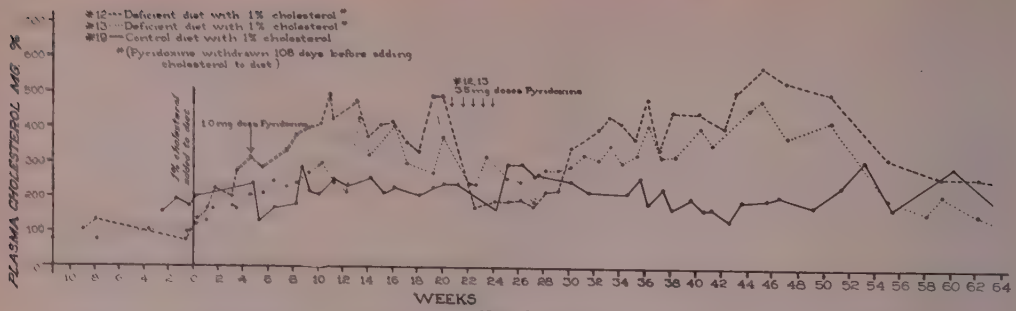

FIG. 1.

Effect of cholesterol ingestion on plasma cholesterol of control and pyridoxine-deficient monkeys.

the cholesterol intake of the latter was 3 to 4 times as great.

The administration of a single mg dose of pyridoxine hydrochloride to the deficient monkeys after 32 days of cholesterol feeding did not appear to have much influence on their levels; on the other hand, when 5 doses (3.5 mg each) of the vitamin were administered twice weekly between the 145th and 158th day of cholesterol feeding, the plasma cholesterol levels decreased soon after starting the vitamin treatment. This occurred even though the food consumption and cholesterol intake increased. The blood cholesterol levels remained low for several weeks and then returned to their former high plane within approximately 6 weeks after the last dose of pyridoxine. Without any alteration in the diet the levels again declined at the end of the 55th week. This might be accounted for to some extent by a decreased food consumption. On the other hand, Weinhouse and Hirsch(6) have made a similar observation after prolonged cholesterol feeding of rabbits and attribute the decline to impairment of absorption of cholesterol from the gastointestinal tract as a result of deposition of lipids in the lining and wall of the stomach and bowel. This explanation could be applicable in the present case.

The second group of monkeys consisted of 4 males weighing 2.01-2.28 kilos. During the course of the cholesterol studies they were fed a basal diet consisting of casein 18, powdered sucrose 59, salts 4, corn oil 2 and hydrogenated cottonseed oil (Crisco) 6. This diet differs from that fed the first group in that it contains a higher percentage of fat. The plasma cholesterol was followed before and after the introduction of 1% cholesterol into the diet. During the latter part of this experiment the deficient monkeys received daily a small suboptimal dose of pyridoxine hydrochloride (10γ per kg). The pertinent information concerning the time of introduction of the cholesterol and the nutritional status of the animals are listed in the table.

Although the second experiment has so far been carried out for a much shorter period, the results observed with the animals in Group II are essentially similar to those observed with the monkeys in Group I. The findings are summarized in Table I. The average plasma cholesterol values for the cholesterol period are higher in both deficient and control monkeys of Group II than they are for the corresponding animals of the first group. This may be accounted for by the fact that the diet of Group II contained a much higher proportion of fat and therefore probably increased the absorption of cholesterol in this group. In spite of the fact that the cholesterol ingestion was 2 to 4 times greater in the control animal his average plasma cholesterol was significantly lower than that of the deficient monkeys.

For the purpose of determining the effect of equalizing the cholesterol intake the latter was adjusted to 0.25 g of the sterol per kilo of body weight for a 6-week period. Cholesterol mixed with a small amount of sucrose and approximately 6 cc of corn oil was ad-

6. Weinhouse, S., and Hirsch, *Arch. Path.*, 1940, v30, 850.

ministered by mouth instead of being incorporated in the basal diet. The same substantial difference in the plasma cholesterol levels was encountered during this regimen. The control animal, no. 38, had an average value of 257, nos. 36† and 37†, 381 and 444 mg % respectively. As might be expected, the average plasma level of no. 38 was lowered somewhat because of a reduction in his intake of cholesterol.

Discussion. The greater hypercholesterolemia induced in pyridoxine deficient monkeys by the feeding of cholesterol is of interest. Whether this results from enhanced absorption or a decline in the ability of the vitamin B6 deficient monkey to handle cholesterol is not known. It is possible that cholesterol may be absorbed more efficiently in the pyridoxine-deprived animal than it is in the control. However, this appears to be contrary to what we have observed in the case of glucose. Evidence obtained in this laboratory with the latter indicates that glucose absorption is much more rapid in the animal supplied with pyridoxine. The fact that glucose absorption is decreased in the pyridoxine deficient animal does not prove that cholesterol absorption will also be retarded since they are 2 entirely unrelated chemical compounds. On the other hand, Gubler *et al.*(7) have obtained evidence which they consider indicative of increased iron absorption in the vitamin B6 deficient rat. The results with the animals in Group I do, nevertheless, show that considerable absorption of cholesterol took place in spite of the fact that the diet was low in fat, containing only 2% corn oil.

It is perhaps pertinent to note that the basal diet fed the animals is essentially cholesterol free. In spite of this, animals which have been maintained on this diet over long periods of time showed 'normal' plasma cholesterol levels indicating that cholesterol synthesis is a normal mechanism in the rhesus monkey.

While our studies are not yet completed, to date, we have not found arteriosclerotic lesions in cholesterol fed monkeys which had received adequate supplements of pyridoxine, and the arteriosclerotic lesions of pyridoxine deficiency do not appear to have been enhanced by the administration of cholesterol.

Summary. Ad libitum feeding of a low or moderate fat diet containing 1% cholesterol to rhesus monkeys resulted in a greater hypercholesterolemia in the pyridoxine deficient monkey than it did in the control monkey. This occurred despite the fact that the cholesterol intake of the control animal was 2-4 times greater than that of the deficient animals.

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† Deficient.

Cortisone and Blood Pressure.* (18566)

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It is now generally accepted that the favorable effects of cortisone and the adrenocorticotropic hormone on the manifestations of many disorders are related to the production of some measure of hyperadrenocorticalism (1). The similarities of Cushing's syndrome and the alterations which ensue after cortisone therapy are noteworthy(2). With the ex-

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TABLE I. Effect of Daily Dose of 100 mg Cortisone on Blood Pressure in Subjects Without and With Renal Disease.

Diagnosis	Avg "resting" B.P. For 10 days before cortisone	Avg "resting" B.P. 10th to 20th day of treatment
	A	
Normal	136/ 83	138/ 84
Rheumatoid arthritis	126/ 85	125/ 85
" "	115/ 74	115/ 77
Normal	113/ 64	116/ 62
Rheumatoid arthritis	110/ 70	110/ 70
Eczema	104/ 76	106/ 75
Rheumatoid arthritis	100/ 66	98/ 70
" "	96/ 60	94/ 61
	B	
Malignant hypertension	170/105	181/115
" "	190/118	215/130
Hypertension with nephrosclerosis	155/103	170/118
Hypertension with nephrosclerosis	160/101	186/120
Chronic glomerulonephritis	185/128	210/160
Nephrotic syndrome	110/ 70	136/ 90
Renal amyloidosis	116/ 80	140/108
Disseminated lupus	122/ 65	138/ 76
" "	136/ 90	150/101
Diabetes with nephrosis	118/ 82	138/ 96

ception of data indicating a depressor effect in uncomplicated hypertensive vascular disease and a rise in blood pressure in Addisonian patients(3), there is little information concerning the production of hypertension by cortisone, a conspicuous feature in Cushing's syndrome. In fact, modification of arterial tension by this steroid has usually been regarded as negligible(4).

The widespread use of cortisone in many clinical conditions prompts a review of this problem in both short and long-term studies.

Results. Table IA summarizes observations on "resting" blood pressure(3) in 8 normotensive patients with repeatedly negative urinalyses who received parenteral cortisone† for short periods while in the hospital. No significant changes in arterial tension were evident in this group.

An additional 25 normotensive ambulatory patients, again with no evidence of renal disease, were treated for rheumatoid and other arthritic disorders for periods of from one to 7 months. Some of these received 50 to 75 mg of cortisone by mouth 2 or 3 times daily, others obtained satisfactory clinical responses by intramuscular injections given from one to 4 times per week with weekly dosages varying from 300 to 600 mg. The blood pressure, recorded casually at approximately weekly intervals, showed no significant alterations beyond those which might be attributed to disappearance of fever or increased physical activity, and remained within normal limits (below 140/90) in all but one subject. This patient, taking 25 mg of cortisone orally 3 times per day, developed some edema and transient hypertension up to 160/100 after one month of therapy, but thereafter the majority of readings were normal although occasional diastolic values of 90 to 95 mm of mercury appeared during the third and fourth months.

Table IB summarizes observations in 10 patients with albuminuria in varying disease states in whom nitrogen retention was not a consistent feature. A rise in "resting" blood pressure was noted in every instance irrespective of the presence or absence of antecedent hypertension. No subject in this group developed congestive failure and, judging from weight changes, fluid balance and hematocrit studies, the occasional signs of mild salt and water retention were comparable to the series of patients without albuminuria.

Discussion. The results would indicate that cortisone exerts little influence on the arterial tension of normotensive patients without renal disease treated for several months with the dosages customarily employed. It remains possible, however, that larger doses or therapy continued for longer periods of time, may yet be associated with hypertension similar to that seen in Cushing's syndrome.

On the other hand, rises in both systolic and diastolic pressures were evident within a

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† Purchased from Merck & Co., Rahway, N. J., through funds supplied by the U.S.P.H.S.

few weeks in the group with renal involvement. Whether kidney damage results in greater retention or chemical modification of administered cortisone, or whether the response of the diseased kidney to the steroid is altered, cannot be determined from the present study. It is of interest that hypertension associated with desoxycorticosterone can be achieved more readily in nephritic rats (5). It is recommended that cortisone be used with greater care in patients with renal disease, particularly if antecedent hypertension is evident.

Conclusions. Modification of arterial tension by cortisone, in the doses generally employed, has been noted but rarely in short- and long-term observations of subjects without renal disease. A rise in blood pressure has been the rule among patients with renal damage of varying types irrespective of the presence or absence of antecedent hypertension.

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Specificities of Hyaluronidases Formed by Several Groups of Streptococci.* (18567)

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In a previous study(1) enzyme-inhibitors appearing in human serums during streptococcal infections could not be accounted for on the assumption that Group A enzymes were type-specific. It appeared that heterotypic Group A strains of streptococci gave rise *de novo* to analogous enzyme-inhibitors. Since then, some experimental observations have been made in regard to the relative specificities of hyaluronidases produced by Groups A, B, C and G strains of streptococci. These studies form the basis for the present report.

Materials and methods. Streptococcal hyaluronidases. One hundred and fifteen strains of streptococci‡ were studied for hyaluronidase activity. Each strain was grown in buffered Todd-Hewitt broth for a period of 15 to 18 hours. The enzyme titer was determined by the mucin clot prevention test of McClean (1,3). Streptococcal hyaluronidases of satisfactory titer (*e.g.* >1:16) were stored in sealed glass ampoules at –70°C. The number of strains in each group studied and the number producing enough enzyme for working purposes appear in Table I.

Enzyme-inhibitor. Hyaluronidases of high activity (titer of 1:128 or more) were derived from 10 strains of streptococci.§ Bacteria-free filtrates were titered and aliquots of ac-

TABLE I. Hyaluronidase Activity of Streptococci.

Group	No. of strains	Enzyme activity <16*	Enzyme activity 16-128	Enzyme activity 128-1024	No. of enzyme producers	% producing enzyme
A	35	27	7	1	8	23
B	7	5	2	0	2	29
C	28	15	10	3	13	46
D	16	16	0	0	0	0
G	28	10	13	5	18	64
K	1	1	0	0	0	0

* MCP test; reciprocal of enzyme dilution.

* Aided in part by a Grant from the Division of Research Grants and Fellowships, United States Public Health Service.

† Trainee, National Cancer Institute.

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‡ Acknowledgement is made to Drs. Rebecca Lancefield, Lowell Rantz, Nobel Sherwood, Elaine Updyke, and Julia Coffey for supplying many of the strains used in the study.

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TABLE II. *Hyaluronidase Inhibitor Titers of Immunized Rabbits.*

Group	Strain	Enzyme-inhibitor titer*
A	Type 3 (Meeks)	128
A	Type 4 (T4 95 Rb5)	128
A	Type 4 (Bois)	256
A	Type 22 (T22 88 3)	64
B	V9	0
C	13	256
C	J14S8 0 3	128
C	B3	64
C	S 57	128
G	88238	256

* Expressed as reciprocal of serum dilution; 16 antigenic units of homotypic enzyme preinoculation serums did not contain inhibitor.

tive enzymes were inoculated into 24 rabbits by intravenous and intramuscular routes. Enzyme-inhibitors in varying amounts, appeared within 2 to 6 weeks. The best enzyme-inhibitor titers obtained appear in Table II. *Enzyme-inhibition.* Quinn's modification of the method described by McClean(2,3) was used. The specificity of streptococcal enzyme-inhibitors was also studied using serial dilutions of serums and enzymes in box titrations. *Antibody absorption.* Cross absorption tests with enzymes and bacterial cells and immune serums derived from several strains of Groups C and G streptococci were made. Equal parts of 1:4 dilutions of an antiserum were separately mixed with enzyme and washed streptococcal cells. The mixtures were placed in a waterbath for one hour at 37°C. and then in the icebox (4°C.) overnight. Enzyme-inhibition tests were made using serial dilutions of serums and enzymes in box titrations. Enzyme and serum controls were included in the tests.

Results. Type specificity of hyaluronidases from Group A streptococci. Among 35 strains of Group A streptococci, 8 produced sufficient hyaluronidase for inclusion in the tests.

The specificity of hyaluronidases obtained from 6 strains of known type was measured by reciprocal enzyme-inhibition reactions. The results of these tests, which appear in Table III, indicated that several heterotypic strains of Group A streptococci produced enzymes with similar characteristics.

Quantitative distinctions may be observed in the reciprocal neutralization of heterotypic Group A strains. These variations do not indicate that there are major differences in enzyme configuration as measured by enzyme-inhibitor, for among rabbits inoculated with identical enzymes and producing, as a rule, inhibitor with heterotypic overlap, several serums inhibited only the parent enzyme.

Specificity of hyaluronidases of Groups A, B, C and G streptococci. Twenty-five strains of streptococci were studied by reciprocal enzyme-inhibition reactions. These tests revealed that Groups A and B enzymes were serologically distinct. Enzymes obtained from Groups C and G strains were serologically related but were unrelated to the Group A or B enzymes.

The serologic distinction of these streptococcal enzymes was explored further in box titrations of enzymes and enzyme-inhibitors. The specificities of Groups A and B hyaluronidases and the serologic overlap of enzymes derived from Groups C and G strains were again observed. Additional tests, with Group specific enzyme-inhibitors and enzymes prepared from Groups A, C and G strains other than those tabulated here, reaffirmed these relationships. Results obtained in one of these tests appear in Table IV.

The immunologic relationships of Groups C and G hyaluronidases were further determined by reciprocal neutralization and absorption. These studies indicated that a single exposure of serums to (a) active enzymes or (b) washed bacterial cells of Group C or G reduced Group C and G enzyme-inhibitor titers, respectively.

Discussion. The specificity of hyaluronidases formed by streptococci has not been explored, except sketchily. McClean(4) prepared enzyme-inhibitors with three Group C strains and found that Group A enzymes were not inactivated by them. Friou(5) observed the failure of human Group A enzyme-

§ Dr. E. L. Updyke, Communicable Disease Center, United States Public Health Service, kindly rechecked the group and type of streptococci used in these tests.

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5. Friou, G. J., *J. Infect. Dis.*, 1949, v84, 240.

TABLE III. Type Specificity of Group A Hyaluronidases.

Serum inhibitor	Reciprocal of serum dilution inhibiting enzyme* action					
	Type 3 (Meeks)	Type 4 (Bois)	Type 4 T4/95/Rb5	Type 4	Type 22 T/22/83/3	Type 22
Type 3 (Meeks)	128	64	128	128	16	32
" 4 (Bois)	32	16	128	16	4	16
" 4 (T4/95/Rb5)	128	128	128	256	0	128
" 4 (101)	32	32	128	32	32	32
" 22 (T22/83/3)	0	0	0	8	64	0

* 16 units of enzyme.

TABLE IV. Group Specific Enzyme-Inhibitor Titers of Groups A, B, C, and G Streptococcal Hyaluronidases.

Antigenic units of enzyme	Final dilutions of group specific enzyme-inhibitors* inactivating indicated enzymes											
	A enzyme-inhibitor				C enzyme-inhibitor				G enzyme-inhibitor			
	A_e	B_e	C_e	G_e	A_e	B_e	C_e	G_e	A_e	B_e	C_e	G_e
2	128	2	0	0	0	0	128	96	128(?)	0	64	64
4	96	0	0	0	0	0	96	128	0	0	24	48
8	64	0	0	0	0	0	32	32	0	0	8	32
16	16	0	0	0	0	0	16	16	0	0	<8	16
32	16	0	0	0	0	0	<8	0	0	0	<8	8
64	0	0	0	0	0	0	0	0	0	0	0	0

A_e = Group A, type 4 enzyme. C_e = Group C, strain 13 enzyme.
B_e = Group B, strain V9 enzyme. G_e = Group G, strain S8258 enzyme.
(?) Insufficient enzyme, control at 1:2 dilution of enzyme gave partial hydrolysis.
* Enzyme-inhibitor specific for Group B enzymes could not be prepared; neither of two strains studied produced enough enzyme.

inhibitors to affect hyaluronidases produced by Group C and G streptococci. The latter observations have failed to define group specificity of hyaluronidases. They do point out, once again, that in the natural history of human streptococcal infections most are caused by Group A strains.

The finding that enzyme-inhibitors prepared from Groups C and G enzymes were serologically similar was of interest because of other relationships found among these groups. Hare(6) found some serums produced by the injection of Group C strains to give cross precipitation with Group G strains; this usually occurred, however, if the animals had been immunized for a longer period than usual to obtain a satisfactory titer. Lancefield(7) observed that Groups C and G streptococci possessed a common protein antigen which was responsible for the confusing cross reactions observed by Hare. It is not suggested here that this protein antigen is related to hyaluronidases produced by Groups C and G streptococci. It may or may not be. We wish only to add at this time that some strains in these Groups shared the distinction of forming hyaluronidases possessing similar antigenic components.

Summary. Hyaluronidases produced by strains of Groups A and B streptococci are serologically distinct. Hyaluronidases produced by strains of Groups C and G are serologically indistinct. Each of the latter is distinctly different from hyaluronidases formed by strains of streptococci, Groups A and B.

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Effects of Intraperitoneally Injected Arginase on Growth of Mammary Carcinoma Implants in the Mouse. (18568)

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Arginine has been reported to influence nuclear activity of cells *in vitro*(1). There are also indications that this amino acid induces mitosis in the normal cells and stimulates such activity in tumor cells grown *in vitro*. Its enzyme however, tends to inhibit this phenomenon (2). There is also evidence that the usable amount of arginase in the animal carcass varies with the sex or that the enzymatic-activator (or deactivator of an inhibitor) is sex-linked(3). Additional effects of intraperitoneally injected arginase have been observed on Hamilton and C_3H strain mice(4). While pursuing another phase of work involving the enzyme arginase it was thought that intraperitoneal injections of arginase into *normal* Swiss mice, and those exhibiting spontaneous adenocarcinomas would be of some interest. Injecting various amounts of enzyme we observed reductions, ranging from 30 to 90%, in the size of existing tumors. A few tumor mice received injections directly into the tumor mass with similar results. Histological examinations of the effected tumors in both direct tumor and intraperitoneal injections showed cytoplasmic and nuclear changes occurring in the tumor cells with increased fibrosis throughout the mass. In many instances the connective tissue response of the tumor resulted with almost entire disappearance of the neoplastic cells.

A preliminary experiment using C_3H strain mice implanted with locally available mammary carcinomas was set up to study the effect of arginase on induced tumors. These implants were made by a trochar in the flank of 30 (mixed) C_3H strain mice. After 11 days the tumors were established and the animals were separated at random into 3 groups of 10 each. Group "A" was designated as control. Group "B" received daily intraperitoneal injections of 1 cc of 0.16 M arginine monohydrochloride in 0.1 M phosphate buffer at pH 7.5. Group "C" received daily intraperitoneal injections of arginase. This solution was obtained by extraction of beef liver and activated with cobalt acetate (1.25 mg Co^{++} per cc) for 3 hours at 40°C. Each injection was 1 cc and contained 60 arginase units(5). Every animal in each group was weighed daily and its tumor measured with calipers. The experiment was terminated at the end of the tenth treatment day. At this time all mice of groups "A" and "B" had died of the tumor, while all animals receiving the arginase (Group "C") were still alive. This was 21 days after successful tumor implantation.

TABLE I.

Group	"A" (Control)	"B" (Arginine IP)	"C" (Arginase IP)
Body wt 11th day after implant	29.5	32.6	31.4
Body wt at necropsy	35.0*	34.8*	34.8
Tumor length 11th day after implant	15.2	21.7	18.8
Tumor length at necropsy	37.1*	38.8*	26.7
Tumor wt at necropsy	6.4*	6.2*	6.1
% growth compared to group "A"	100	104.5	72.0
Ratio tumor length/wt expressed in %	100	107.9	74.1

* Animals died at various intervals after 11th day of implant. Length (L) expressed in mm. Wt (W) expressed in g. Growth refers to length increase in mm. IP—intraper.

Table I presents the average tumor growth from day of implant, per cent growth as compared to group "A", and ratio of tumor

1. Irons, W. G., *Dental Research*, 1946, v25, 180.

2. Bach, S. J., and Lasnitzki, J., *Enzymologia*, 1947, v12, 198, 205.

3. Wiswell, O. B., *Science*, 1950, v112, 117.

4. Irons, W. G., and Wiswell, O. B., *Science*, 1947, v106, 393.

5. Mohamed, M. S., and Greenberg, D. M., *Arch. Biochem.*, 1945, v8, 349.

length to weight in percent of each group over the 10 day treatment period. A liver factor other than arginase might possibly have been responsible for this growth phenomena; however, it is known that the procedure for arginase extraction and purification from beef liver produces a substance of relative high purity(5). Therefore it was assumed that the active factor could have been arginase.

Summary of results. 1. Animals receiving arginase treatment did not die of their implanted tumors during the 21-day period. The controls (Group "A") and those receiving arginine (Group "B") died within this period. 2. Those animals receiving arginine indicate a tumor length weight ratio increase of 7.9% over group "A". 3. The group receiving arginase (Group "C") exhibited a 28% decrease in size as compared to the controls in group "A". 4. The ratio of tumor length to weight was 26% lower in the group receiving arginase. 5. Tumor specific volume (tumor length to weight) is offered as an accurate tumor measurement method.

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Ability of Infants to Taste PTC: Its Application in Cases of Doubtful Paternity. (18569)

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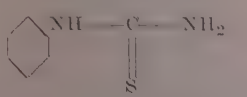
In 1930 while working with phenyl thio carbamide (PTC), Fox(1) observed that the compound was tasteless to some individuals but extremely bitter, even to the point of nausea, for others. Approximately 40% of the subjects tested were "taste blind" to this material. He investigated a number of chemically similar substances and concluded that the characteristic taste of PTC and related compounds was due to the C = S linkage, *viz.*:



He postulated that this difference must be an inherent property of the individual's saliva, a concept recently proved by Cohen and Oydon(2). The observations of Fox were promptly confirmed and extended by others. Snyder(3) studied 440 individuals from 100 families and observed an incidence of 68.5% tasters (T) and 31.5% non-tasters (t), and Blakeslee(4-6) investigating 103 families, encountered 66.5% tasters and 33.5% non-tasters. The ability to taste PTC appeared to follow Mendelian laws, tasting being an inherited as a dominant characteristic and non-tasting as a recessive. Thus, the union of two tasters invariably produced tasters and the union of non-tasters produced all non-tasters. The union of a taster and a non-taster may produce both tasters and non-tasters, but if the offspring is a taster, at least one parent must be a taster.

It occurred to us that the ability to taste PTC might profitably be applied in cases of doubtful paternity. Before doing so, however, it would be necessary to establish: (1) that the ability to taste PTC was differentiated at an early age and (2) that it could be satisfactorily determined by an objective test. The observations of Fisher, Ford and Huxley(7) on chimpanzees indi-

1. Fox, A. L., *Proc. Nat. Acad. Sci.*, 1932, v18, 115.

2. Cohen, J., and Oydon, D. P., *Science*, 1949, v110, 532.

3. Snyder, L. H., *Science*, 1931, v74, 151.

4. Blakeslee, A. F., *Proc. Nat. Acad. Sci.*, 1932, v18, 120.

5. Salmon, T. N., and Blakeslee, A. F., *Ibid.*, 1935, v21, 78.

6. Blakeslee, A. F., and Salmon, T. N., *Ibid.*, 1935, v21, 84.

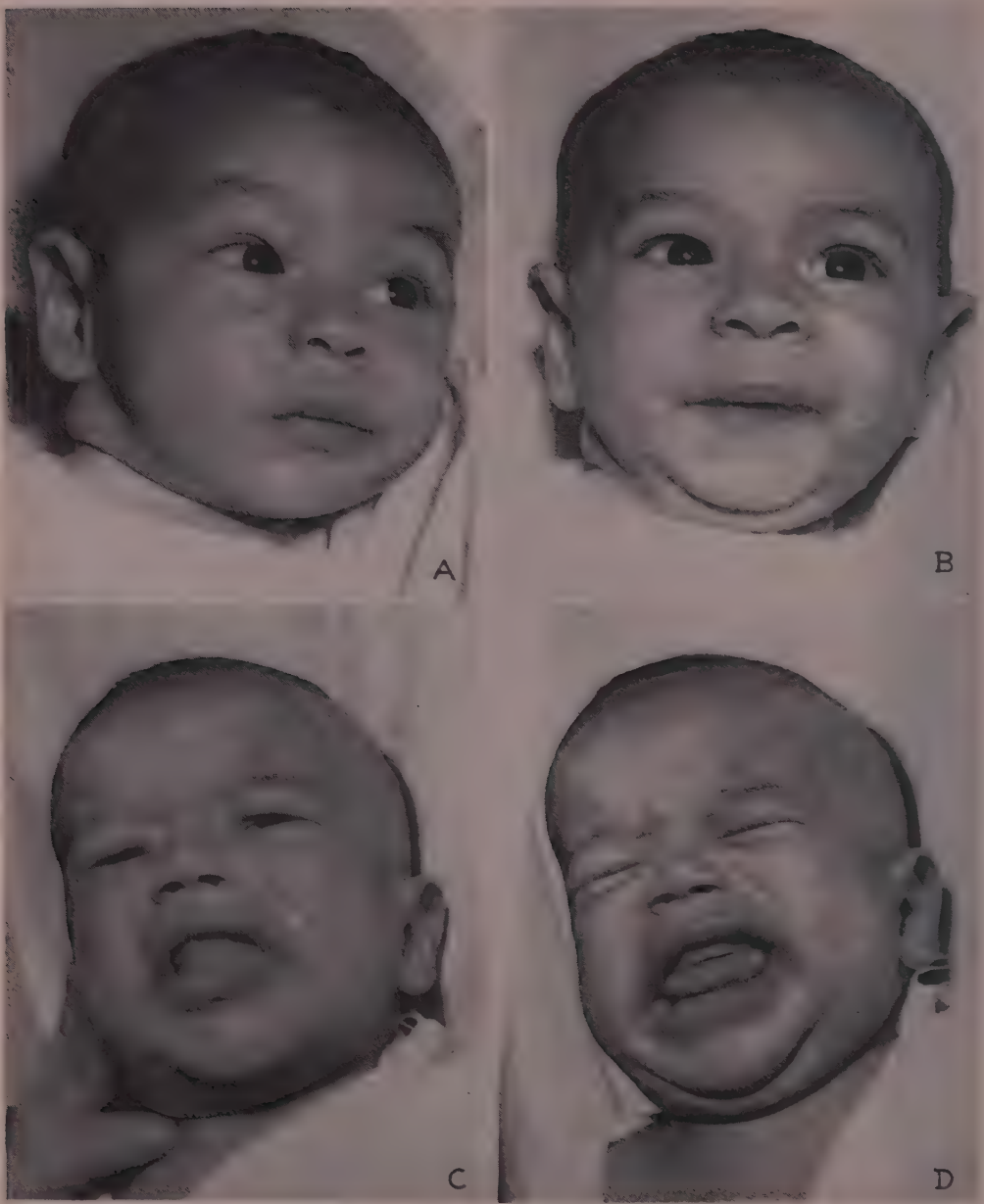


FIG. 1.
Response of infants to phenyl thio carbamide. A—negative; B—1+ (suggestive); C—2+; D—3+.

cated that tasters and non-tasters could readily be identified in these animals and encouraged us to undertake similar observations on infants.

By instillation of 0.5 cc of a dilute solution of PTC with a medicine dropper between the

[7] Fisher, R. A., Ford, E. B., and Huxley, J. H. Nature, 1939, v154, 505.

lips of infants it was found that certain infants showed no response (Fig. 1A), whereas others showed a definitely unfavorable response which could be graded + to ++++ according to its severity: 1+ indicating merely salivation or a few "mouthing" movements (Fig. 1B), 2+ definite grimacing (Fig. 1C), 3+ a reaction which began with salivation, progressed to grimacing and ended with crying (Fig. 1D) and 4+ when retching was added to the picture. Reactions from ++ to ++++ were considered positive. Each test was repeated several times and controlled by the administration of distilled water in similar manner. It was surprising how readily the ability to taste PTC could be detected by this means even in premature infants. The dilution of PTC employed is a matter of importance in making such tests, since the ability or inability to taste is not absolute, the percentage of tasters increasing when concentrated solutions are used; only a small percentage of individuals give no taste reaction when PTC crystals are placed directly upon the tongue. Blakeslee(4) recommends a dilution of 1:5,000 for routine testing and Riddell(8,9) a 1:20,000 dilution. In the studies here reported a 1:10,000 dilution was employed. Although it is known(5) that variations of 4- to 10-fold may occur in the threshold of individuals tested upon different occasions, the data in the literature testify to the conclusion that such a dilution is satisfactory for dividing the world of tasters from that of non-tasters. In addition to demonstrating the ability of the young infant to taste PTC we were interested in determining whether the ratio of tasters to non-tasters was similar to that reported in adults. Statistical calculations indicated that a sample of 93 babies would have to be studied in order to obtain significant results. This number of infants was accordingly tested. Of these 18 were premature, 37 were full term infants under one week of age and 38 were full term infants ranging from one week to 2 years of age. There were 43 males and 50 females in the group. The racial distribution was too uneven for any analysis.

TABLE I. Response of Infants to 1:10000 Dilution of Phenyl Thio Carbamide.

Age group	Tasters		Non-tasters	
	No.	%	No.	%
Prematures, 1-7 days	11	61.1	7	38.9
Full term, <1 wk	23	62.2	14	37.8
1 wk-2 yr	23	60.5	15	39.5
Total	57	61.3	36	38.7

Results. The results shown in the accompanying table indicate that 57 infants (61.3%) were tasters and 36 (38.7%) non-tasters. Age did not appear to affect the distribution. The incidence of tasters among the males (60.5%) was similar to that among the females (62%).

Discussion. The results indicate that the ability or inability to taste PTC is present at birth, and can be demonstrated by using a 1:10,000 dilution of the drug. By this procedure the ratio of tasters to non-tasters in infants was found to be similar to that reported in adults.

The practical value of this test in establishing paternity or non-paternity would appear to be the situation in which the child is a taster and the mother a non-taster, a situation which may be expected to occur in about 20% of cases of disputed paternity. Only a tasting male could be the father of such a child.*

Summary. Phenyl thio carbamide is known to divide the adult population into 2 groups, those capable of tasting its intense bitterness (T) and those to whom it is tasteless (t). The capacity for tasting or failing to taste the compound is inherited according to Mendelian laws. It is demonstrated that the ability to taste or not to taste this compound is demonstrable in early infancy and it is suggested

8. Riddell, W. J. B., *Trans. Ophth. Soc.*, 1939, v59, 275.

9. Riddell, W. J. B., and Wybar, *Nature*, 1944, v154, 669.

10. Boyd, W. C., *Science*, 1950, v112, 153.

* At the time this work was undertaken we were not aware that any medicological application of the ability to taste PTC had been made. However, it has recently come to our attention that evidence of this kind was employed in France. Lamy, M., Pognan, C., Schweissguth, O., and Fauvert, D. Exhibit at 6th International Pediatrics Congress, Zurich, July 1950.

that such data can be profitably employed in cases of doubtful paternity in situations where a tasting child is born to a non-tasting mother.

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The Use of I^{131} Red Cell Plasma Ratio as a Measure of Thyroid Function.* (18570)

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Chemical analyses have shown that the normal chloride content of the red blood cells is approximately 190 mg % and that of plasma approximately 365 mg %(1). This gives a ratio of 0.51 for $\frac{\text{RBC chloride}}{\text{plasma chloride}}$. Similar analyses of cells and plasma in persons treated with bromides give a similar ratio for RBC bromide/plasma bromide(2). It has been noted that chloride, bromide and iodide pass rapidly across the red cell membrane *in vitro* and there is no reason to doubt that equilibrium is reached rapidly *in vivo,* as well(3). Rall *et al.* have shown from *in vitro* studies in human blood that the red cell plasma ratios for iodide and chloride were 0.67 and 0.59, respectively(3). As would have been expected from a consideration of the chemical similarities and mean ionic radii of iodide as well as of chloride and bromide ions, we have noted from *in vitro* studies that iodide ion does give an RBC/plasma ratio similar to that of the other two halides, namely about 0.5(4,5). It has been well established that the degree of conversion of inorganic iodine to protein-bound iodine and the appearance of the latter in the blood stream is an index of thyroid activity. I^{131} has been used as a tracer by a number of investigators to follow protein-bound iodine levels of the plasma and its relationship to types of thyroid disease(6,7). In the past, it usually has been necessary to employ laborious analytical methods to obtain a reliable separation of the protein-bound iodine in the plasma from the inorganic iodine fraction.

In the studies to be described in this report, it was found that inorganic iodine penetrated the red cell rapidly. However, the cell membrane apparently is relatively impermeable to large protein molecules containing labeled iodine. Therefore, the concentration of iodine in red cells, using I^{131} as a tracer, may be used as an index of the inorganic iodine content in the blood. The red cell activity will, therefore, indicate how much of the total plasma activity is due to inorganic iodine and how much is protein bound. The varying red cell plasma ratio at different times following a test dose of I^{131} is the result of the organic binding of I^{131} by the thyroid and/or the removal of protein-bound iodine from

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† Division of Radiology, University of California School of Medicine, San Francisco and Crocker Laboratory, University of California, Berkeley.

‡ Stanford University School of Medicine, San Francisco.

1. Everett, Mark R., Medical Biochemistry, 2nd Ed. New York 46, Paul B. Hoeber, Inc., P. 585.

2. Everett, Mark R., Medical Biochemistry, 2nd Ed. New York 46, Paul B. Hoeber, Inc., P. 614.

3. Rall, J. E., Marschelle, H. P., and Albert, A., Proc. Soc. Exp. Biol. and Med., 1950, v74, 460.

4. Jacobs, M. H., *Ann. N. Y. Acad. Sci.,* 1950, v50, 825.

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7. Friedburg, A. S., Ureles, Alvin, and Hertz, Saul, Proc. Soc. Exp. Biol. and Med., 1949, v70, 679.

the blood stream by the body.

Methods. Clinical evaluation: Carrier-free I^{131}, as supplied by the Isotopes Division, U. S. Atomic Energy Commission, Oak Ridge, Tenn., was given orally in 50 cc of water as sodium iodide with a small amount of $NaHSO_3$ (.015 N base). Test doses given ranged from 100 to 150 microcuries. The patients described in these studies were diagnosed upon clinical grounds. The diagnosis was confirmed with the usual laboratory tests indicated in the diagnosis of thyroid disease. This included an estimation of the basal metabolic rate, blood cholesterol level in patients suspected of hypothyroidism, and thyroid uptake studies using I^{131}, as well as the blood studies cited here. Larger amounts up to 10 millicuries were administered for therapy. It should be noted that the exact quantity given did not alter the ratios observed. The separation of red cells and plasma was made by centrifuging 5 cc samples of heparinized blood at 3000 r.p.m. for 30 minutes and transferring 2 g portions of red cells and plasma into separate dishes. A few drops of 95% ethyl alcohol were added to each sample to aid in obtaining a flat film which is necessary for consistent geometry. The samples were dried at 75°C for 8 to 24 hours to remove moisture. It was found that I^{131} was not lost by this procedure. After assay of the activity with a G.M. counter, corrections were made for self-absorption. The data are expressed as counts per second. The counting efficiency was approximately 10% of the total number of disintegrations. The ratio of the I^{131} in the red cell/I^{131} in the plasma is not influenced by losses of iodine from the blood either by thyroid uptake or urinary excretion since *in vivo* studies have shown that the equilibrium between the plasma and the red cells is rapidly established. Therefore, a change in the red cell-plasma ratio is a reflection of the presence in the blood of iodine-bound into large molecules which are not able to penetrate the red cell membrane. Presumably the prosthetic groups of these large molecules are iodine-labeled thyroxine and diiodotyrosine. The rate at which these iodine containing molecules are built up in the plasma as well as the total amount present has been related to the synthetic ability of the thyroid(8). For this reason the rate which the red cell, plasma ratio deviates from the value seen with inorganic iodine as well as the total deviation is an expression of thyroid function and tissue utilization of I^{131} tagged materials.

Results. The initial red cell ratios taken 1 to 3 hours after oral I^{131} administration represent inorganic iodine red cell plasma ratios in all groups which presumably was too brief a time interval for measurable amounts of protein-bound iodine to appear in the blood stream. The ratios of euthyroid and hyperthyroid were similar and varied from 0.42 to 0.52 and showed no initial relationship to the thyroid state of the patient. However, with the passage of time the ratios were observed to drop to values as low as 0.05 this particular value being observed at 80 hours after I^{131} administration to a patient suffering from extreme hyperthyroidism. A ratio of 0.05 suggests that only twice this concentration of inorganic iodine is in the plasma and therefore the plasma activity is 90% due to protein-bound iodine. Using the methods described above, blood samples from 13 patients have been studied with the following results.

Two of the patients judged to be hypothyroid showed little or no reduction of their inorganic ratio during the time interval studied which was 80 and 140 hours. The data obtained upon the hypothyroid patients gave half-times of greater than 100 hours when expressed as percent of the original ratio. The meaning of half-time for these purposes is merely an arbitrary expression of the time required for the ratio to drop from its initial value of 0.5 to 0.25. A ratio of 0.25 suggests that one-half of the iodine in the blood stream is bound into molecules which are too large to penetrate the red cell membrane as does inorganic iodine.

Similar studies upon 4 euthyroid patients demonstrated the ability of their thyroids to return greater amounts of the I^{131} test dose

8. Chaikoff, I. L., and Taurog, Alvin, *Ann. N. Y. Acad. Sci.*, 1949, v50, 377.

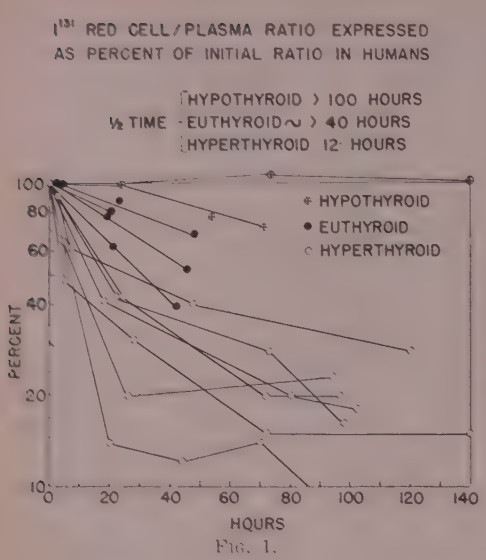

Fig. 1.

to the blood stream in organic combination, with a half-time of about 40 hours. Individual variations approached both hypo and hyperthyroid states. Seven hyperthyroid patients had red cell-plasma ratios which were lower than normal and appeared to be related to the degree of hyperthyroidism. Similar ratios were obtained upon hyperthyroid patients whether they were given test doses of I^{131} or therapeutic doses for the treatment of their disease during the periods reported here. These data are summarized in Fig. 1. It is noted that the ratios of hyperthyroids and euthyroids show greatest variation from each other 24 hours after I^{131} administration.

For this reason, a blood sample taken at this time, after a test dose of I^{131}, should give adequate information with respect to the rate of formation of protein-bound iodine and its deviation from normal. Blood samples from persons suspected of hypothyroidism gave more fruitful information when taken 35 to 75 hours after I^{131} administration. This is due to the fact that euthyroid individuals need this period of time to show a rise of I^{131} protein-bound iodine after a test dose. After successful I^{131} therapy, 2 patients previously having red cell-plasma ratios consistent with hyperthyroidism gave ratios which fell in the normal group when repeat test doses of I^{131} were administered. Further information concerning the I^{131} content of red cells, plasma and thyroid at various time periods in single patients which were typical examples of hyperthyroidism, euthyroidism, and hypothyroidism, is shown in Fig. 2. Such data were obtained on all of the patients described here in order to calculate their ratios.

In hyperthyroidism, I^{131} rapidly disappeared from the plasma reaching a minimum in the case shown at 8 hours after its administration. The initial drop in plasma concentration is due to accumulation by the thyroid and by excretion. Following this, a rise in plasma iodine was observed which was related to the I^{131} administered and represents protein-bound iodine in part. The curve of red cell activity also shows a drop at first but does not show a rise until several hours after the plasma begins to rise. This rise suggests that the lag between the plasma concentration and the red cell concentration represents the time needed for the catabolism of the labeled hormone, after which the iodine returns to the plasma as inorganic iodine and is capable of diffusing into the red cell. The hyperthyroid curves shown in Fig. II, depict thyroid metabolism in the exaggerated state. Normal thyroid metabolism as illustrated in Fig. 2 shows a less rapid disappearance of I^{131} from both red cells and plasma than in the hypothyroid. In addition, no increase in I^{131} activity of red cells or plasma was observed in the normals and hypothyroids during the time period for which they were studied. Iodine was lost at a decreasing rate from the red cells and plasma with the passage of time. This change in rate has also been noted by Myant *et al.*(6).

The fate of I^{131} with respect to red cells and plasma of a clinically myxedematous patient is also shown in Fig. 2. It may be noted from the curves that the I^{131} concentration of both red cells and serum fall off at what is practically an exponential rate and are parallel throughout their course. These curves indicate the excretion of iodide ion as would be expected in the absence of a functioning thyroid. No hormone is formed and the

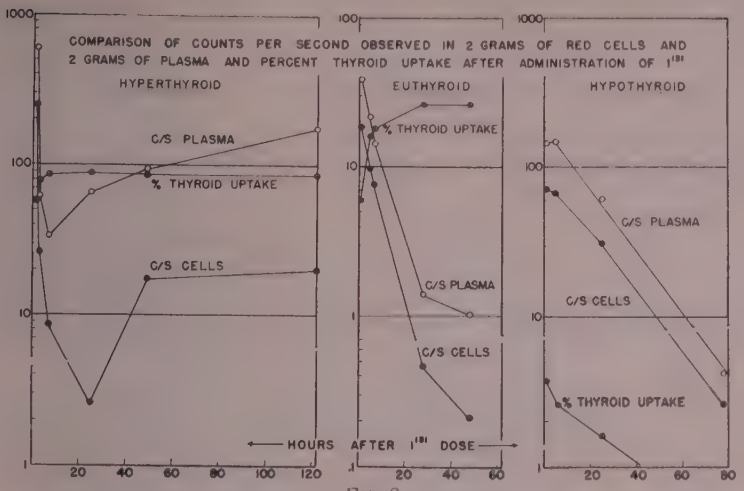

Fig. 2.

Distribution of I^{131} in thyroid and blood of patients which were hyperthyroid, euthyroid, and hypothyroid following oral administration.

relative concentrations of iodine in red cells and plasma is related to their essential iodide space. As was previously described, the ratio was found to be about 0.5 throughout the time of study.

In conclusion, we believe that, due to its simplicity, this method has more to offer than external measurements of I^{131} uptake by the thyroid or determination of I^{131} urinary output, in the diagnosis of thyroid disease. The red cell-plasma relationship illustrates not only the disappearance of inorganic iodine from the blood stream but its reappearance as protein-bound iodine as well.

Summary. 1. The ratio of red cell space for inorganic iodine to that of plasma was found to be about 0.5, in both *in vitro* and *in vivo* studies. 2. The initial red cell-plasma ratio was the same in hyperthyroid, euthyroid, and hypothyroid patients immediately after the oral administration of a test dose of I^{131}. 3. The ratio dropped as the inorganic iodine test dose was incorporated into larger molecules by the thyroid and returned to the blood stream. 4. The drop in red cell-plasma ratio was due to the relative impermeability of the red cell to large iodine-containing molecules. 5. The rate of drop of the red cell-plasma ratio is an expression of thyroid activity.

We are indebted to Joseph G. Hamilton, Crocker Laboratory and School of Medicine, University of California, Berkeley and San Francisco; and R. R. Newell, Stanford University School of Medicine, for their counsel and stimulating criticism; and to Earl R. Miller, School of Medicine, University of California, for consultation on the choice of patients.

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Effect of Cortisone upon Chondroitin Sulfate Synthesis by Animal Tissues.* (18571)

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The symptoms and the pathological picture in arthritis and certain other of the so-called "collagen diseases" appear to be closely related to an abnormal increase in the amount of extracellular connective tissue substance in the lesions. Responses to cortisone therapy are usually marked by a decrease in the amount of ground substance in the lesions. Ragan and co-workers(1) have shown that cortisone inhibits the formation of granulation tissue in wounds. We have shown(2,3) that wound granulation tissue *in vitro* and *in vivo* exhibits high capacity for the incorporation of inorganic sulfate into the chondroitin sulfate of the tissue. Considering these facts, it appeared to us that cortisone might exert its action through the inhibition of the synthesis of certain mucopolysaccharides by connective tissue cells. If this should be the case, one might expect cortisone to cause a decrease in the synthesis of chondroitin sulfate from inorganic sulfate. We have found(4) that the labeled inorganic sulfate fixed by tissues maintained *in vitro*(2,3) or *in vivo*(5) is bound in the chondroitin sulfate of the connective tissue. Using a method similar to that of Meyer and Rapport(6), it was found that the action of hyaluronidase upon the labeled sulfate containing material was very similar to its action upon the chondroitin sulfate from bovine connective tissue. It appeared to us that the labeled sulfate was bound in the chondroitin sulfate of the connective tissue ground substance.

Since the *in vitro* method of studying anabolic sulfate metabolism lends itself to quantitative determinations, it was considered desirable to use the method to study the influence of cortisone upon synthesis of chondroitin sulfate by tissues.

Experimental. Preliminary experiments with chickens had indicated that dosages of cortisone acetate approximating 25 mg per day per kilo of body weight had no effect upon wound healing. This was in marked contrast to the effect in rats where we found that 8 mg per day per kilo almost completely suppressed the formation of granulation tissue. Since most of our quantitative data were for chicken tissues, it was considered desirable to continue with this species. The experimental procedure used in this investigation has been described in detail in previous papers(2,5,7). Briefly, it is as follows: Replicate samples of tissue weighing approximately 3.0 mg each were prepared from the heart, skeletal muscle, and liver of chick embryos on the sixteenth day of incubation. Samples were also prepared from the regenerating tissue of healing muscle wounds of young adult chickens. The granulation tissue was removed from sterile wounds on the fifth day following partial section of *M. pectoralis* major. Each sample was placed in 2 ml of sterile medium† containing sodium sulfate and graded concentrations of recrystallized cortisone alcohol.

* The technical assistance of Gladys Friedler, Angela Congiundi and Doris Frankel is acknowledged. The radioactive sulfur used in this investigation was supplied free by the United States Atomic Energy Commission. This investigation was supported by grants from the American Cancer Society upon recommendation of The Committee on Growth of The National Research Council. The cortisone was supplied by Merck and Co.

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† Tyrode's solution modified to contain 4.8 mg of sulfate ion per liter of solution. The sulfate was labeled with radioactive sulfur, S^{35}, to give a specific activity of 6.5×10^7 counts per mg of sulfate ion as determined by a method described earlier(5).

TABLE I. Effect of Cortisone Alcohol Upon Chondroitin Sulfate Synthesis and Sulfate Esterification by Embryonic Chick Tissues *in Vitro*.

Tissue	Conc. of cortisone in medium, mg/l	Sulfate fixed by tissues (μg/100 mg tissue): Calculated as SO_4 ion	Calculated as chondroitin sulfate synthesized	Wt of SO_4 ion esterified (μg/100 mg tissue)
Skeletal muscle	0	1.3	5.85	.45
" "	35	.32	1.4	.28
" "	70	.15	0.68	.20
Heart ventricle	0	1.41	6.3	.53
" "	35	.45	2.0	.33
" "	70	.22	1.0	.23
" "	120	.10	0.45	.10

TABLE II. Effect of Cortisone Acetate and Cortisone Alcohol Upon Chondroitin Sulfate Synthesis and Sulfate Esterification by Tissue from Healing Wounds in Chickens.

Conc. of cortisone in medium	mg/l	Sulfate fixed by tissues (μg/100 mg tissue): Calculated as SO_4 ion	Calculated as chondroitin sulfate synthesized	Wt of SO_4 ion esterified (μg/100 mg tissue)
Controls, none	0	1.4	6.3	.70
Susp. of cortisone acetate	200	1.2	5.4	.60
Cortisone alcohol	25	1.2	5.4	.60
" "	50	.50	2.3	.40
" "	75	.30	1.4	.20
" "	120	.08	0.36	.07

Tissues were removed on the fifth day after wounding.

In order that we might observe the effect of cortisone upon growth and survival of tissues *in vitro,* control cultures of embryonic heart tissues were prepared in Carrel flasks. The tissue fragments were placed in plasma clots and covered with bovine serum ultrafiltrate containing embryo extract and concentrations of cortisone alcohol equivalent to those used for the chemical studies. Heart, skeletal muscle, and wound tissues were incubated at 37°C for 45 hours. Liver tissue samples were incubated at 21°C for 70 hours in order to obtain maximum conjugation of the sulfate in the medium (7). At the end of the incubation period the tissues were removed from the medium and soaked for 20 minutes in cold tap water. The washings were combined with the culture medium, and the soluble labeled organic sulfate in the medium was determined by the method of Layton and Frankel (7). The labeled sulfate fixed in the tissue was determined by the author's method (5).

Results. By observing the tissues in Carrel flasks it was found that high concentrations of cortisone did not affect the migration of fibroblasts during the first 72 hours. Rapid degeneration of fibroblasts occurred after the third day. Pulsation of heart tissues continued for more than 2 weeks in the presence of cortisone. It would appear that the tissues were capable of carrying on essential metabolic activities while maintained in concentrations of cortisone which suppressed chondroitin synthesis.

It was found that cortisone caused a graded inhibitory response in the fixation of labeled sulfate by embryonic tissues. The sulfate fixation was almost completely suppressed by cortisone concentrations in excess of 100 mg per liter. The esterification of sulfate by heart and skeletal muscle was inhibited to

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TABLE III. Effect of Cortisone Alcohol Upon Sulfate Conjugation by Embryonic Chick Liver Tissue *in Vitro*.*

Conc. of cortisone in medium, mg./l	No. of samples	Wt of sulfate ion esterified (μg. 100 mg tissue)
0	4†	7.1
50	12	5.2
100	12	4.5

No phenol was added to the medium(7).

approximately the same degree as was the fixation. Data from a representative experiment are shown in Table I.

From the data of Table II it will be seen that tissue from healing wounds was similar to embryonic tissue in its response to cortisone. Conjugation of sulfate by liver tissue was not affected by concentrations of cortisone which completely suppressed sulfate fixation and synthesis of soluble organic sulfate by heart and skeletal muscle (Table III).

Sulfate conjugation by the liver tissue is probably a detoxication mechanism(7-9) and hence a phase of the catabolic metabolism. The fact that cortisone did not affect the synthesis of ester sulfate by liver tissue, while it did inhibit esterification of sulfate by heart and skeletal muscle tissues may indicate that different mechanisms are involved. It is possible that the soluble organic sulfates synthesized by heart and muscle are related to the connective tissue and plasma mucoids and to heparin. The decrease in the sedimentation rate of the blood of patients receiving cortisone therapy may be due to a decreased synthesis of soluble plasma mucoids by the connective tissue cells.

It is possible that the system affected by cortisone therapy is the one which synthesizes the chondroitin sulfate moiety of the mucoids. These investigations have been extended to a preliminary study of tissues *in vivo*. Similar results were obtained. These will be reported in a subsequent paper.

Summary. 1. The experiments described indicated that cortisone inhibits the synthesis of chondroitin sulfate by embryonic and wound tissues maintained *in vitro*. Graded responses were obtained with graded concentrations of cortisone in the medium. Cortisone was shown to inhibit the synthesis of soluble organic sulfate by heart and skeletal muscle; it had no such effect upon the synthesis of soluble ester sulfate by liver tissue. 2. Concentrations of cortisone which suppressed sulfate fixation, had no apparent effect upon the initial migration of fibroblasts. Cortisone had no effect upon the pulsations of heart tissue. 3. It was suggested that the palliative action of cortisone in the connective tissue diseases may be due to its inhibitory effect upon the synthesis of the chondroitin sulfate moiety of the connective tissue ground substance.

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On the Existence of a Cell Granule in a Thermophilic Bacterium.* (18572)

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In a study of a thermophilic bacterium† (1-3) we showed that most of the enzymes

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† Thermophile No. 2184 obtained from the National Canners Association. This organism has tentatively been identified by us as *Bacillus stearothermophilus* (Donk).

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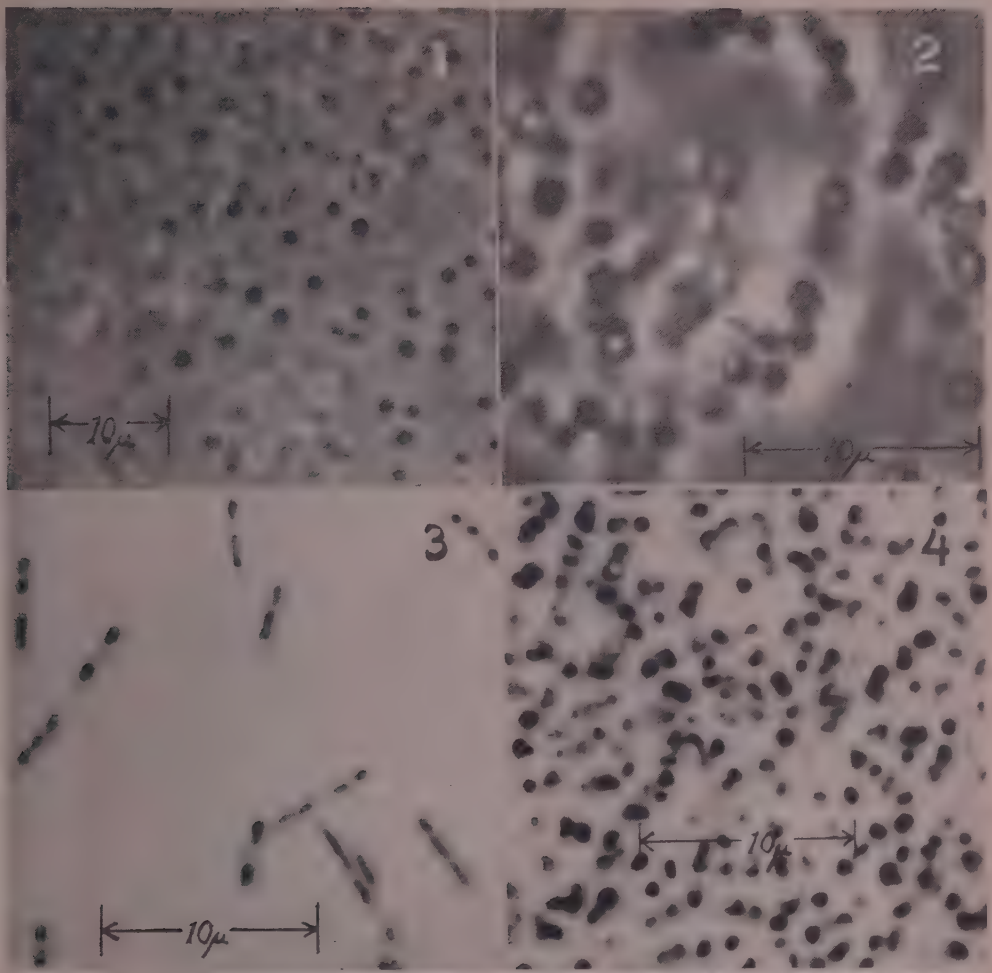

FIG. 1. Wet mount of particles liberated from [illegible] after treatment with lysozyme. Photographed with phase contrast microscope.

FIG. 2. Wet mount of washed red fraction photographed by phase contrast.

FIG. 3. Eight hour agar slant culture of [illegible] using hydrochloric acid-Giemsa stain.

FIG. 4. Red fraction stained with hydrochloric acid-Giemsa.

were associated with an insoluble fraction called the red fraction. This fraction separated on centrifugation from the lysate of the bacteria which had been treated with lysozyme. We stated that the red fraction behaved as though it were a specific particle of bacterial protoplasm. The belief has now been supported by data from several independent sources. The data supporting the conclusion follow.

The most direct evidence is that obtained with the phase contrast microscope. Fig. 1 is a picture of a saline suspension of bacteria a few minutes after they have been treated with lysozyme. The cells have disappeared leaving nothing visible but spherical bodies. These bodies can be separated by centrifugation and compose the fraction referred to as the red fraction. Fig. 2 is washed red fraction photographed at greater magnification. In both figures there is a striking similarity in the size and shape of the granules. Brown-

ian movement hampered the photography of these preparations, and, as a result, only a fraction of the total number of particles was in focus at any one moment.

The size of the particles observed in Fig. 1 and 2 is greater than the size of particles we have observed with the phase microscope in the intact bacterial cell. (No illustrations showing the granules in whole cells were attempted because of motility.) This difference in size does not mean that the released particle could not be the same as that in the cell. It has been noted before that inclusion bodies swell and change their shape once released from their native state. Mitochondria, for instance, change shape easily depending upon the medium in which they are suspended, a fact observed many years ago by Bensley and Hoerr(4) and recently explored by Harman(5).

The question may be raised whether the granules might not be spores, which this organism is capable of producing, rather than protoplasmic particles. The particles seen here are definitely not spores. Spores are highly refractile in wet mounts and are not opaque to light as are the red fraction granules. Further, the bacteria were grown in liquid media at 65°C with copious aeration, conditions which did not favor spore formation. Spores, when they are formed, can be observed by the usual spore staining technics. They appear much larger than do the granules of the red fraction. Granules, when packed together during centrifuging, have a bright red color due to cytochrome c and cytochrome oxidase. This is not likely to be a characteristic of spores.

Further evidence is gained by staining the bacteria and the red fraction. When the Giemsa stain is applied to the bacterial cell preceded by a brief treatment with N hydrochloric acid at 60°C(6), bodies can be seen which could easily be red fraction granules. Fig. 3 shows these intensely stained bodies within whole cells of thermophile No. 2184. Fig. 4 is a preparation of red fraction similarly stained.

TABLE I. Oxygen Uptake at 37°C of Red Fraction in Presence of Succinate and Other Substrates.

Preparation	μl oxygen/hr
Red fraction	12
" " + succinate (.18 M)	110
" "	0
" " + p-phenylenediamine (.018 M)	450
Red fraction + hydroquinone (.018 M)*	252
Red fraction + p-phenylenediamine + cyanide (5×10^{-4} M)	87
Red fraction + hydroquinone + cyanide (5×10^{-4} M)*	0

Warburg flasks contained the following: 1.0 ml phosphate buffer pH 7.4; .5 ml fresh red fraction preparation containing 26 to 32 mg protein; .5 ml or distilled water to make a total volume of 2.8 ml.
* Corrected for autoxidation of hydroquinone.

From Fig. 3 and 4 it follows that the liberated granules are larger than the particles appearing in the intact cells. Roughly this difference is about 2-fold, which is about what was observed with phase microscopy. It is also apparent that the granules in the stained preparations (Fig. 4) are smaller than the granules seen in the wet mounts (Fig. 2). This is due to shrinkage upon drying during the preparation of the stained mount.

Another line of evidence that the particles are specific granules comes from their enzyme activity. Like mitochondria(7), the granules are the site of considerable enzyme activity. Thus far, we have reported on malic dehydrogenase, cytochrome oxidase, and apyrase(1-3). Malic dehydrogenase, cytochrome oxidase, and cytochrome c were found to be concentrated in the granules of the red fraction. In addition to these, there are aldolase(8), adenosinetriphosphatase(9), cytochrome b and succinoxidase. Figures from a typical run for the activity of the latter two are given in Table I. The cytochrome b assay is based on the manometric method rather than on the

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spectrometric, since the strong cytochrome c bands seem to prevent observation of other cytochromes.

Of special significance is succinoxidase activity. The oxidation of succinate by molecular oxygen requires a system of enzymes and cytochromes that seems to demand an unbroken organization(10). The opinion is held by many(11) that a disturbance of such a close association as is present in the cell results in destruction of succinoxidase activity. If this be true, then any preparation retaining succinoxidase activity must have been removed from the cell as an intact unit. The succinoxidase system from most sources, by virtue of its demand for organization, is extremely sensitive to inactivation(11). We have found the system in the red fraction granule to be sensitive to drying and aging and treatment with acetone, although not especially vulnerable to heat.

Taking all of the evidence together, from phase microscopy, from staining technics, and from enzyme activity, we now feel safe in making the statement that the red fraction is a specific granule isolated from the bacterial cell. To the best of our knowledge this is the first such instance reported for bacteria.

Conclusions. The red fraction, previously isolated from a stenothermophilic bacterium, represents a specific piece of bacterial protoplasm. The evidence presented consists of photographs taken with the phase contrast microscope, photomicrographs of stained preparations of bacteria and of red fraction, and of enzymatic data. The enzymatic data rest chiefly on the presence of succinoxidase activity.

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Effect of Desoxycorticosterone on the Colon: Its Relation to the Action of Cation Exchange Resins in Man.* (18573)

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One of the actions of the adrenal gland affects the transfer of sodium from the lumen of the kidney tubule to the surrounding blood stream. Desoxycorticosterone acetate (DOCA) acts in a similar fashion. This system of sodium transfer found in the kidney is analogous to that found in the intestinal tract where sodium is again transferred from the lumen to the surrounding circulation. Previous investigation of the influence of the adrenal gland on the transfer of sodium in the latter system has indicated that the adrenalectomized animal transfers sodium across the wall of the intestine at a somewhat slower rate than normal(1-3). The present study concerns the influence of DOCA on the mucosa of the colon as indicated by the eventual excretion of sodium in the stool of rat and man.

The rat was selected because normally sufficient amounts of sodium appear in the stool to measure changes with certainty(4,5). In man, the fecal excretion of sodium is normally so small(6) that changes are often difficult to interpret. For the purpose of this study, a cation exchange resin was fed to

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man to insure sufficient amounts of sodium in the stool so that changes could be quantified(7). After suitable periods of observation DOCA was administered to both rat and man, and the changes in the fecal excretion of electrolytes were measured.

Procedures. Three Rockland County white rats weighing 400 to 500 g were studied in separate metabolism cages. They were allowed food and distilled water *ad libitum*. The diet consisted of Purina checkers, containing 150 mEq of sodium and 250 mEq of potassium per gram. Each day, the rats were weighed and their daily intake of food and water recorded. Stools obtained over 3-day periods were pooled for analysis. Electrolyte excretion in the stool of these rats was measured over a 36-day period: 12 control days, 12 days during which each rat received 1 mg of DOCA in sesame oil daily, and 12 recovery days. In the studies conducted in the human, the subjects selected were ambulatory hospital patients without evidence of disturbance in their fluid balance. They were fed the regular ward diet, containing 80 mEq of potassium and allowed salt *ad libitum*. In addition, they received 100 mEq of sodium chloride daily in the form of nonenteric coated tablets. Ammonium carboxylic resin† was administered in dosage of 15 g 3 times daily for a period of 27 days. For a 9-day period, from the 10th to the 18th day of the 27 days on resin, the subject received daily intramuscular injections of DOCA in sesame oil. Urine was collected in 24-hour specimens, and the stool in 72-hour specimens throughout the period of observation. The stools of both rat and man were dried under infra red light. Triplicate aliquots of the dried feces were treated with concentrated sulfuric acid and ashed overnight at 700°C. The ash was dissolved in hydrochloric acid

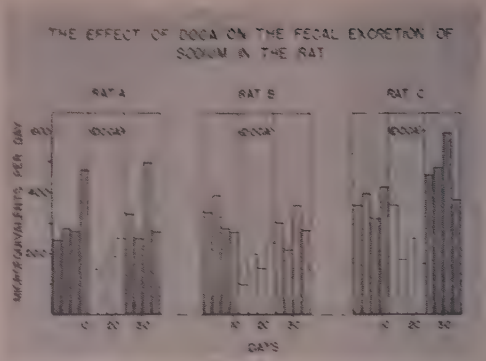

Fig. 1.

and analyzed for sodium and potassium. The urine collected in the human studies was analyzed for sodium and potassium. Sodium and potassium were determined with an internal standard flame photometer(8).

Observations. Observations in the animal. The rats consumed about 16 g of food daily and did not alter their salt or water intake appreciably throughout the experimental period. Rats receiving 1 mg of DOCA daily evidenced an appreciable decrease in their fecal excretion of sodium. Comparing the mean fecal sodium excretion during 12 days control with the 12 days on DOCA, Rat A showed a 44% decrease, Rat B showed a 46% decrease, and Rat C showed a 35% decrease while on DOCA (Fig. 1). During the 12-day DOCA period, there was a tendency for the fecal sodium to return towards normal values. This is quite probably due to a depression of the rats' endogenous DOCA-like activity. Fecal potassium was also measured in these rats. Comparing the mean fecal potassium excreted during the 12-day control period with that excreted during the 12 days of DOCA administration, Rat A decreased 15%, Rat B decreased 13%, and Rat C increased 9%. These changes are within the daily variation of fecal potassium excretion and are not considered significant.

Observations in man. To investigate the influence of DOCA on the absorption of sodium from the intestine in man, a cation

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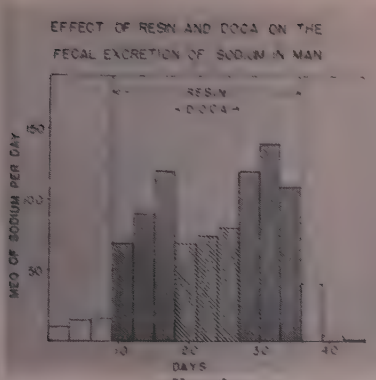

Fig. 2.

exchange resin was fed. The subject was a 55-year-old male suffering from central nervous system lues. When he was fed 45 g of ammonium carboxylic resin daily, his fecal excretion of sodium increased to a mean of 68 mEq a day for the first 3-day collection period (Fig. 2.). In the second 3-day period, the fecal sodium reached 90 mEq daily, and in the third increased to 120 mEq per day. At this point DOCA was administered intramuscularly for 9 days in doses of 15 mg daily for the first 3 days, 20 mg daily for the next 3 days, and 25 mg daily for the last 3 days. The fecal excretion of sodium decreased from 120 mEq per day to 68, 73 and 80 mEq in the 3 successive 3-day collection periods while the subject was receiving DOCA. When the DOCA was discontinued, the fecal excretion of sodium again increased to a mean of 120, 140 and 116 mEq per day in 3 successive collection periods. The resin was now discontinued, and in the ensuing 9 days, fecal sodium fell to normal values.

The urinary sodium averaged 280 mEq a day during the 9-day period prior to the administration of resin, and when resin was introduced, fell to a mean of 150 mEq a day during the first 9 days on resin. There was a further fall in urinary sodium to a mean of 87 mEq a day for the 9 days when DOCA was administered in conjunction with the resin. When DOCA was discontinued, urinary sodium increased to a mean of 141 mEq daily, and when resin was discontinued increased to a mean of 272 mEq daily, values comparable to those obtained during the similar periods prior to the administration of DOCA.

The fecal excretion of potassium increased from 50 to 70 mEq per day when DOCA was administered in addition to the resin and returned to 50 mEq in the post-DOCA period (means of 9-day periods). The increase in fecal potassium excretion is small and is not considered to be a direct effect of DOCA on exchanging sodium for potassium but rather, attributed to an increase in available space on the resin as a consequence of the decrease in sodium. This view is substantiated by the observations in the rat where resin was not used, since under these circumstances, DOCA did not augment fecal potassium excretion.

The urinary potassium progressively decreased while resin was administered and did not seem to be influenced by DOCA. This progressive decrease in urinary potassium excretion is presumably due to progressive losses of potassium in the stool(7).

Similar studies were conducted in 2 other patients with similar results. In one, the fecal excretion of sodium was 50, 62, and 66 mEq of sodium daily in 3 successive collection periods on resin. With the addition of DOCA the fecal excretion of sodium fell to 3, 23, and 13 mEq daily in 3 successive collection periods. In the second patient on resin, only 13 to 19 mEq of sodium appeared in the stool but with the addition of DOCA the fecal excretion of sodium again fell to 6 mEq daily.

Discussion. DOCA decreases sodium excretion in the stool of the rat and when man is fed exchange resin, DOCA decreases the effectiveness of the resin in removing sodium in the stool. The action of cation exchange resin in the intestine is better understood when consideration is given to the mechanism of transfer of sodium across the wall of the gut. The exchange of ions on the surface of the resin is almost immediate and the pattern of electrolytes on its surface is dependent on the number and nature of electrolytes in the surrounding media(9,10). As the concentra-

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tion of sodium varies along the intestinal tract, the amount of sodium fixed to the resin changes, the exchange occurring as soon as the surrounding electrolytes change. When the content of the ileum reaches the colon, the resin is suspended in a solution containing about 140 mEq of sodium per liter(11). In the colon, sodium moves more rapidly from the lumen to the blood than from the blood to the lumen which results in a net reabsorption of sodium from the colon(12). As the sodium ion leaves the lumen of the colon a new equilibrium is established between that sodium on the resin and that in the colon contents. It is the balance of these two attractions, the resin and the colon mucosa, which eventually determines the effectiveness of cation exchange resins in removing sodium in the stool.

Summary. The present data indicate that the action of DOCA is not limited to a control of the transfer of sodium from the lumen of the kidney tubule to the surrounding blood stream but also influences the transfer of sodium from the lumen of the intestine to the surrounding blood stream. DOCA, in addition, influences the transfer of sodium through the sweat and salivary glands by decreasing the sodium concentrations in their secretions(13,14). The evidence indicates that DOCA affects the transfer of sodium through various organs, among them the colon, in each instance limiting the escape of sodium from the body.

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Bilateral Cortical Necrosis of Kidneys in Cortisone-Treated Rabbits Following Injection of Bacterial Toxins.* (18574)

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Thomas and Mogabgab(1) reported that treatment with cortisone or ACTH produced a striking modification of the local skin reaction of rabbits to the intradermal injection of Shwartzman's meningococcal toxin. The treated animals did not develop the usual reaction of edema and erythema caused by toxin; instead, numerous petechial hemorrhages appeared throughout the skin site approximately 24 hours after the intradermal injection of toxin. The observations suggested that cortisone and ACTH, while inhibiting the local inflammatory response to toxin, might have brought about an increase in the vulnerability of the tissue to direct damage by bacterial toxin. Similar experiments, briefly described elsewhere(2), were undertaken to determine the effect of cortisone on the course of skin infection by living microorganisms, employing various strains of Group A hemolytic streptococci which were known to produce local inflammatory reactions, sometimes followed by abscess formation, in normal rabbits. When cortisone was administered for 2 or 3 days before and after an injection of

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these microorganisms, little or no local reaction occurred in the skin but the rabbits regularly developed extensive bacteremia and died within the next 4-12 days. Colony-like deposits of streptococci were observed in the interstitial tissue of the heart and kidney in some of these animals(3).

In the light of the observed increase in susceptibility to the systemic effects of living microorganisms which was brought about by cortisone, it became of interest to learn whether treatment with this substance would alter the reaction of internal organs to bacterial toxin. Accordingly, rabbits were treated with cortisone and then given an intravenous injection of toxin derived from meningococci or *Serratia marcescens*, with accompanying control animals receiving cortisone alone or toxin alone. The present paper is concerned with a description of the kidney lesions which developed in cortisone-treated rabbits after a single intravenous injection of bacterial toxin.

Materials and methods. Albino male and female rabbits weighing 1.5-2.0 kg were obtained from a single breeding stock for these experiments, and were maintained on a diet of Purina rabbit pellets. Cortisone, supplied through the generosity of Merck and Company was injected intramuscularly as a suspension in 1.0 cc sterile saline. In most experiments the rabbits were given cortisone in a daily single dosage of 25 mg for 4 days, and toxin was injected in the marginal ear vein on the third day. In some experiments, indicated below, the treatment period was shortened or the daily dosage of cortisone was decreased. Meningococcal "agar washings" toxin(4) filtered free of bacteria, was kindly supplied by Dr. Gregory Shwartzman, of the Mount Sinai Hospital, N. Y. This material was given intravenously in the marginal ear vein, in a dose of 2 cc of 1-10 dilution in saline. A partially purified "polysaccharide toxin" from *Serratia marcescens*, similar to that employed in experimental tumor therapy (5), was supplied by Dr. Murray Shear, of the National Institutes of Health, Bethesda, Maryland. The latter material, designated as "P-25 toxin", was given intravenously in an amount of 0.4 mg contained in 2 cc saline.

The rabbits were divided into 5 groups and subjected to treatment in the following manner: 1. Thirty-six rabbits were treated with 25 mg cortisone per day for 4 days, and injected intravenously with meningococcal toxin on the third day. 2. Thirty rabbits were given the same amount of meningococcal toxin intravenously, without cortisone treatment. 3. Thirty rabbits were treated with 25 mg cortisone daily for 4 days, and received no toxin. 4. Twelve rabbits were treated with 25 mg cortisone daily for 4 days, and received an intravenous injection of Shear's P-25 toxin (0.4 mg) on the third day. 5. Twelve rabbits received the same dosage of P-25 toxin as above, and were not treated with cortisone. In addition, smaller groups of rabbits were treated with cortisone in lower dosage or for shorter periods of time prior to the injection of bacterial toxin.

Results. In the cortisone-treated rabbits which were given intravenous meningococcal or P-25 toxin (Groups 1 and 4), kidney lesions occurred within 24-72 hours which had the gross and microscopic characteristics of bilateral renal cortical necrosis. The incidence of these lesions is indicated in Table I, in

TABLE I. Bilateral Renal Cortical Necrosis in Cortisone-Treated Rabbits Following an Injection of Meningococcal or *S. marcescens* Toxin.

Group	Exp. procedure		No. of rabbits	No. with bilateral renal cortical necrosis
	Cortisone treatment	Bacterial toxin		
I	25 mg/day for 4 days	M.t.* 3rd day	36	19
II	0	M.t.	30	0
III	25 mg/day for 4 days	0	30	0
IV	25 mg/day for 4 days	P-25 t. 3rd day	12	10
V	0	P-25 t.	9	0

* Meningococcal toxin.

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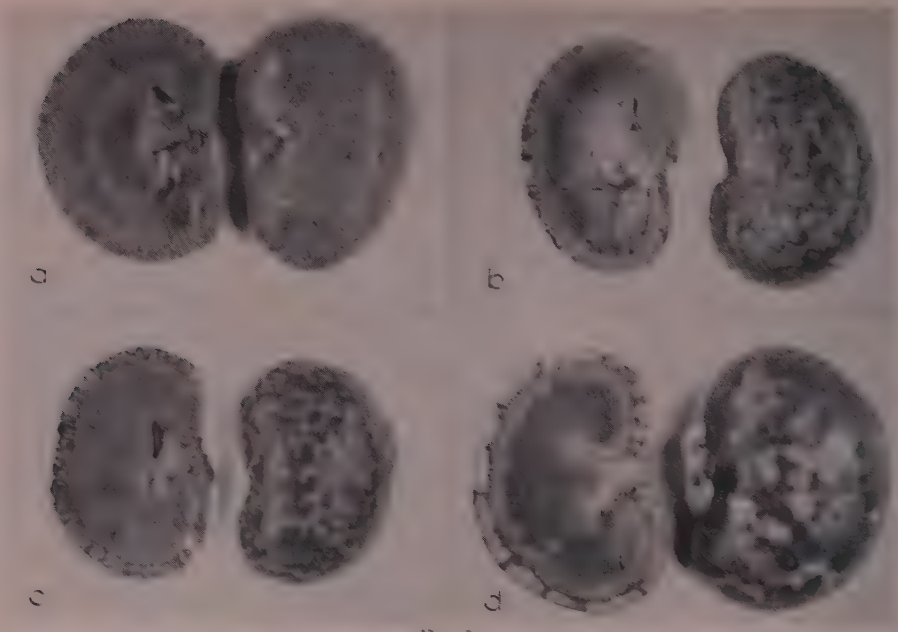

FIG. 1.
Gross appearance of kidneys of 4 cortisone-treated rabbits 48 hr after an intravenous inj. of meningococcal endotoxin.

which it will be seen that 19 of 36 cortisone-treated rabbits receiving meningococcal toxin, and 10 of 12 similar animals receiving P-25 toxin, exhibited bilateral cortical necrosis. In 30 control rabbits treated with cortisone alone, and in 42 receiving toxin alone, renal necrosis did not occur.

The gross appearance of the kidneys in the rabbits given both cortisone and toxin is illustrated in Fig. 1, in which photographs of the surface and cut edge of the kidneys of 4 representative animals are shown. Multiple deep red or purple areas of punctate hemorrhage, and white, pin-head sized areas of necrosis were observed throughout the renal cortices. In general, little or no involvement of the medulla was grossly discernible. Microscopically, the lesions were marked by severe tubular necrosis, interstitial hemorrhages, and many hyaline thromboses in the glomeruli. Fig. 2 illustrates the demarcation of necrosis and hemorrhage within the cortex (Fig. 2a), and the extent of tubular necrosis and hemorrhage (Fig. 2b). A more detailed account of the pathology in these animals will be given in a later communication. Variability was noted in the extent of hemorrhage and necrosis in different rabbits receiving the same doses of cortisone and toxin. The most severe lesions were observed in animals which died spontaneously within 24-72 hours after the injection of toxin. Animals which survived and seemed well at the end of the 3 day period showed less extensive cortical hemorrhage and necrosis, although the lesions were easily seen by gross inspection.

In all rabbits given cortisone in a dosage of 25 mg daily for 3 or more days, whether or not toxin was also administered, gross abnormality of the liver was observed. This organ was larger than normal and extremely friable; microscopically the hepatic cells showed marked enlargement and cytoplasmic vacuolization. In some of the rabbits treated with cortisone alone, the kidneys were somewhat larger and paler than normal and showed, on microscopic examination, generalized dilation of the tubules throughout the cortex. No tubular necrosis or hemorrhages were seen in microscopic sections of these kidneys.

In the group of control rabbits given menin-

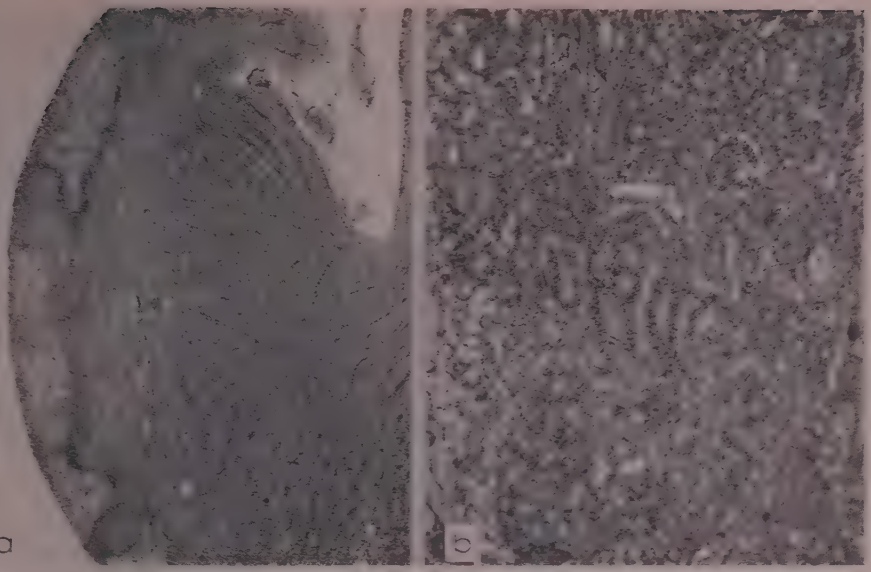

FIG. 2.

Microscopic appearance of kidney of a cortisone-treated rabbit 48 hr after an intravenous inj. of meningococcal endotoxin.

gococcal or P-25 toxin alone, some animals appeared sick and ruffled for several hours after injection and a few (less than 10%) died within 4-12 hours with no demonstrable tissue lesions. The remainder of the group survived without apparent ill effect and, when sacrificed 3 days later, exhibited no gross or microscopic abnormalities in the kidney, liver or other organs.

The minimal effective dose of cortisone for the production of renal necrosis in combination with toxin has not yet been accurately determined. In preliminary experiments, the lesions occurred in 2 of 6 rabbits which received 5 mg of cortisone daily for 4 days prior to the injection of P-25 toxin. In 6 animals the effect of shortening the period of pretreatment with cortisone was studied. These animals received 25 mg of cortisone for 3 days, and P-25 toxin intravenously on the second day; 5 of the 6 rabbits developed bilateral cortical necrosis of the kidneys within 24-72 hours after toxin. When cortisone was limited to a single injection of 25 mg on the day of injection of toxin, or 24 hours later, no renal lesions were observed.

Cultures of the blood and kidney tissue of 12 rabbits with renal necrosis were made, and all were negative. No instances of a similar kidney lesion were demonstrable in rabbits subjected to other, unrelated experimental procedures, including more than 50 animals which died following treatment with cortisone and experimental infection with hemolytic streptococci(2,3).

Similar hemorrhagic necrosis of the renal cortex was produced in 7 of 8 cortisone-treated Syrian hamsters, following intraperitoneal injection of Shear's P-25 toxin. These animals were treated for 4 days with 10 mg cortisone daily, and 0.2 mg of toxin was injected on the third day. Hamsters given cortisone alone, or toxin alone, showed no kidney lesions. A more detailed report of the experimental disease in hamsters will be included in a later communication.

Discussion. It is known that bilateral cortical necrosis of the kidneys may occasionally be produced in rabbits when an intravenous injection of gram-negative bacterial toxin is followed, after 24 hours, by another intravenous injection of similar material(4). The development of this lesion has been regarded as the characteristic feature of the "general-

ized Shwartzman phenomenon", because of the requirement that 2 separate injections be given with an intervening period of 24 hours. Black-Schaeffer(6) reported that the same renal lesion could be produced by repeated intravenous injections of meningococci in heavy doses. Apitz(7) reported that a single injection of meningococcal toxin occasionally produced bilateral cortical necrosis in pregnant rabbits. In normal, non-pregnant animals, single injections of meningococcal or *S. marcescens* toxin do not produce kidney lesions regardless of the dose employed or the length of time allowed to elapse following injection.

It is possible that the development of renal cortical necrosis during treatment with cortisone may be analogous to the change in the local skin reaction to meningococcal toxin which was noted earlier(1). In both instances, treatment with cortisone results in hemorrhage and necrosis when toxin is injected—locally when the latter is given intradermally, and in the kidneys when injected intravenously. The possible relation of these reactions to the Shwartzman phenomenon, in which changes in the susceptibility to tissue proteolysis or in the function of polymorphonuclear leucocytes have been postulated (8,9), is a subject for further investigation.

It should be noted that the doses of cortisone which were employed in these experiments were greatly in excess of those which have been used in the therapy of human disease. Until adequate information is available concerning the minimal effective amount of cortisone, no implications are warranted as to the possible effects of such therapy in humans. At this time the phenomenon is of interest because it provides a new experimental model for investigating both the action of cortisone and the mechanisms of tissue damage by gram-negative bacterial toxins.

Summary. 1. Bilateral cortical necrosis of the kidneys was observed in rabbits which were treated with large doses of cortisone and then given a single intravenous injection of meningococcal or *S. marcescens* (P-25) toxin. This lesion did not occur when animals were given injections of cortisone alone, or toxin alone. 2. A similar renal lesion occurred in cortisone-treated Syrian Hamsters following an intraperitoneal injection of P-25 toxin.

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Effect of Hypophysectomy on Electrolyte and Water Metabolism in the Dog.*† (18575)

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The adrenalectomized animal exhibits severe disturbances in electrolyte and water metabolism as well as in carbohydrate and protein metabolism. The hypophysectomized animal with marked adrenal cortical atrophy also has profound disturbances in organic metabolism. The purpose of the present in-

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‡ Dazian Foundation Fellow (1949-1950); Porter Fellow of the Am. Physiol. Soc., (1950-1951).

§ Dazian Foundation Fellow.

vestigation was to determine whether the hypophysectomized dogs concurrently exhibit disturbances in water and electrolyte metabolism. The response to loads of water and potassium and to sodium restriction was observed. Glomerular filtration rate and renal plasma flow were measured in an effort to evaluate the role played by alterations of these functions in the inorganic metabolism of hypophysectomized dogs.

Methods. Fifteen hypophysectomized female dogs were used in the various phases of this study. The operative technic,‖ aftercare, and diet were described previously(1,2). The response to insulin was determined in the post-absorptive state in each dog according to a previously described technic(2). This was taken as a measure of the impairment of carbohydrate and protein metabolism. Glomerular filtration rate, effective renal plasma flow, Tm_{PAH} and $Tm_{Glucose}$ were measured in lightly restrained, unanesthetized dogs in the post-absorptive state according to standard technics(3,4). Each renal function value in the subsequent tables represents the average of 3 or more consecutive periods. Water loads consisted of 40 ml per kg body weight, administered by stomach tube to well hydrated dogs. The per cent of this load excreted during the subsequent 3 hours was determined. Sodium restriction was achieved by the administration of a diet that furnished 1 to 2 mEq sodium daily ("Lonalac", prepared by Mead Johnson and Co.).¶ The dogs were maintained in metabolism cages on this diet for 3 weeks. Sodium output was measured in 24-hour urine collections while serum sodium concentrations were determined at intervals. The last serum sodium determination was made on the last day of salt restriction. Potassium chloride or thiosulfate was delivered intravenously at rates that varied between 0.31 and 0.98 mEq per minute for different experiments. After 1½ hours, four 15 minute urine collections were made. Arterial blood was obtained at the mid-point of each collection period. Potassium in the plasma and urine as well as glomerular filtration rate were measured in these studies. In order to facilitate tubular excretion of potassium(5) 3 of the 4 dogs studied were given 4 g potassium chloride twice daily by mouth for 3 to 5 days prior to the experimental day. Sodium and potassium concentrations were measured on an internal standard flame photometer.

To date, post-mortem examination of 5 of the animals used in this study has been completed. The operative area was studied by examination of serial sections of a block consisting of a portion of the sphenoid bone, the tissue occupying the region of the sella turcica and the hypothalamus. The adrenal glands were weighed after 24 hours of fixation in formalin and then multiple sections of each gland were prepared and examined(6).**

Results. Response to insulin. Six weeks or more postoperatively all the hypophysectomized dogs exhibited maximum sensitivity to the hypoglycemic action of insulin(2).

Anatomy. Examination of the serial sections of the "pituitary areas" of the 5 dogs

TABLE I. Effect of Hypophysectomy on Average Renal Functions of 5 Dogs.

Status	Glomerular filtration rate, ml/min.	Renal plasma flow, ml/min.	Filtration fraction, %
Control	68.1	250	27.2
Hypophysectomized	19.0	74	25.8

‖ We would like to thank Dr. A. C. Bratton, Jr., of Parke, Davis and Co. for the supply of Topical Thrombin which was most helpful in controlling hemorrhage during the operation.

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** All anatomical studies were done by Dr. Nathan Lane of the University Hospital, New York University-Bellevue Medical Center.

TABLE II. Effect of Hypophysectomy on Excretion of Water Load in the Dog.

No. of dogs	Status	% of water load excreted in 3 hr		Glomerular filtration rate (ml/min.)
		Avg	Range	
5	Control	101	85-113	66.3
	Hypophysectomized	28	11- 45	20.3
8	"	32	5- 47	18.5*

* Measured in 6 dogs only.

that have come to autopsy to date proved that each was completely hypophysectomized. The anatomical findings of these dogs (K-3, K-8, E-1, E-3, S-5) are reported in detail in a separate paper(6). Neither adenohypophyseal cells (pars distalis, pars tuberalis and pars intermedia) nor cells of the pars nervosa were found. The adrenal glands of all the dogs studied were markedly reduced in weight and showed atrophic changes of all the cortical layers, including the zona glomerulosa.

Renal functions. Several renal functions were measured in 5 dogs before and 6 to 14 weeks after hypophysectomy, at which time the animals showed maximum sensitivity to insulin. In confirmation of other workers (7) glomerular filtration rate and effective renal plasma flow were reduced to approximately one-third or one-fourth of normal (Table I). Filtration rate was also consistently reduced in 8 additional dogs used in the potassium and water load studies (Tables II and IV). Maximum ability of the tubules to excrete p-aminohippurate was measured in 2 dogs, and maximum ability to reabsorb glucose in one. These functions were also reduced to approximately one-third or less of normal following hypophysectomy. No consistent effect on filtration fraction occurred. Four dogs (K-3, E-1, E-3 and S-5) of this group were proven by histological study to be completely hypophysectomized.

Excretion of water load. Observations on the 3-hour urine excretion following the standard water load of 40 ml/kg were made in 5 dogs before and 6 to 11 weeks after hypophysectomy. Eight additional dogs were studied only after hypophysectomy (7 to 49 weeks post-operative). Multiple observations were made on the 13 dogs. The normal dog in water balance excretes approximately 100% of the standard water load within 3 hours(8). The ability of the hypophysectomized dog under identical conditions to excrete the water load was significantly reduced in each instance (Table II). The filtration rate was reduced at the time of observation in each of the 8 hypophysectomized animals in which this function was measured. The reduced excretion of the water load in the operated animals was most likely not due to decreased gastro-intestinal absorption of water since the stomach was empty 40 minutes after the administration of the water load and the animals never showed diarrhea. Three dogs (K-3, E-1 and E-3) proven by histological study to be completely hypophysectomized were part of the group subjected to water loads.

TABLE III. Effect of Severe Sodium Restriction for 3 Weeks on Serum Sodium Concentration in Six Hypophysectomized Dogs.

	No. of observations	Serum sodium concentration (mEq/liter)	
		Avg	Range
Control	12	141	136-147
During salt restriction	17	141	133-147
Last day of salt restriction	6	141	134-147

Sodium restriction. Six hypophysectomized dogs were maintained on a diet that furnished 1 to 2 mEq sodium daily for 3 weeks. Extremely low sodium concentrations were achieved in the urine, the average concentration for the 6 dogs being 0.3, 0.4, 0.5, 2, 4 and

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TABLE IV. Amount of Potassium Filtered by Glomeruli and Excreted in Urine During Infusion of Potassium in Hypophysectomized Dog.

Dog No.	Status	Potassium (microeq/min.) Infused	Filtered	Excreted	Glomerular filtration rate (ml/min.)	Range plasma potassium during infusion (mEq/liter)
S-1	Control	612	285	318*	51.9	5.0-5.9
	Hypophysectomized	368	96	168*	14.1	6.4-7.1
E-1	"	510	221	161	24.7	8.7-9.4
E-3	"	612	137	178*	17.2	7.8-8.4
E-5	"	306	389	386	43.1	8.8-9.3

* Tubular excretion of potassium is demonstrated in those instances where the urinary excretion of potassium exceeds the amount filtered.

4 mEq per liter. The dogs' general condition did not change during the period of salt restriction and body weight was maintained within narrow limits. In spite of the severe sodium restriction, serum sodium levels showed no significant trends (Table III). These observations were made 12 to 33 weeks after operation. Two dogs (E-1 and K-8) proven by histological study to be completely hypophysectomized were included in the group.

Potassium administration. Large doses of potassium were administered intravenously to 4 hypophysectomized dogs. One dog was studied before and after operation. All but dog E-1 received oral potassium for 3 to 4 days before observation. This dog received potassium chloride intravenously, the remainder potassium thiosulfate. The amounts of potassium infused, filtered by the glomeruli and excreted in the urine are summarized in Table IV. Excretion of potassium by the renal tubules is demonstrated whenever the amount of potassium in the urine exceeds the amount of potassium filtered. This phenomenon occurred in two of the hypophysectomized dogs that received potassium pre-treatment and the thiosulfate salt intravenously. The third dog treated in this fashion exhibited almost equal rates of potassium filtration and urinary excretion. Perhaps because of the reduced filtration rate in the operated animals, plasma potassium levels increased during the potassium infusions somewhat more in the hypophysectomized dogs than in normal animals given comparable amounts. Nevertheless, the retained ability of the renal tubules to excrete potassium appeared to be a factor in the protection of the hypophysectomized dog against excessive increases in plasma potassium concentration. Indeed, dog S-1 excreted in the urine almost the same proportion of administered potassium after hypophysectomy as she did before operation, while dog E-5, whose filtration rate was not reduced as much as the others excreted more potassium in the urine than was administered. Dogs E-1 and E-3 were proven by histological study to be completely hypophysectomized.

Discussion. To date post-mortem studies have been completed in 5 of the 15 dogs. All 5 were found to be completely hypophysectomized. Since the remaining dogs showed the same maximum sensitivity to insulin as the animals proven to be completely hypophysectomized, they too probably will prove to be "completely" or "nearly completely" devoid of adenohypophyseal cells(6). Thus the hypophysectomized dog that manifests severely disturbed carbohydrate and protein metabolism and considerable reduction in some renal functions, retains, despite atrophic changes of all layers of the adrenal cortex(6), the capacity to conserve sodium and excrete potassium in a manner comparable to the normal dog. However, the hypophysectomized dog does exhibit a pronounced impairment of the ability to excrete a water load. The reduced filtration rate may contribute to the impaired ability of the hypophysectomized dog to excrete a water load, and may perhaps assist the animal to withstand salt deprivation. In contrast, the adrenalectomized dog with approximately the same degree of impairment of filtration rate is unable to withstand salt restriction.

The available evidence does not permit a conclusion as to the nature of the hormonal

factors which enable the hypophysectomized animal to maintain adequate electrolyte metabolism. Perhaps the hypophysectomized dog, in spite of the lack of adrenocorticotrophic hormone, produces a sufficient amount of adrenocortical steroids to conserve sodium and excrete potassium but not enough to handle water properly. A further possibility would be that the adrenals of the hypophysectomized dog can produce a "salt regulating" but not a "water regulating" hormone. An alternative hypothesis might be that a complete lack of adrenocortical steroids in the hypophysectomized dog is counterbalanced by the absence of one or more pituitary hormones.

The effect of the anterior pituitary growth hormone upon water metabolism in the hypophysectomized dog is the subject of one of the papers of this series.

Summary. Study of hypophysectomized dogs that manifest (a) atrophic changes in all three cortical layers of the adrenals, (b) severe disturbances in carbohydrate and protein metabolism and (c) reduced renal functions (glomerular filtration rate, renal plasma flow and tubular transfer maxima) reveals that: (1) their ability to excrete an orally administered water load is impaired, (2) their ability to withstand sodium restriction is normal and (3) their renal tubules retain the ability to excrete potassium and the animals are able to handle potassium loads almost as well as the normals.

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Effect of Growth Hormone on Water Metabolism in Hypophysectomized Dogs.* (18576)

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During the course of a study of the effects of growth hormone on carbohydrate metabolism in hypophysectomized dogs(1-3) it was observed that the animals developed a severe polyuria. Since there was no glycosuria and the specific gravity of the urine was very low, the increased water exchange appeared comparable to that seen in experimentally induced diabetes insipidus. The polyuria disappeared after cessation of growth hormone administration. These observations suggested that the hypophysectomized dog's impaired ability to excrete a water load(4,5) might, at least in part, be due to lack of growth hormone.

The present investigation deals with the study of the effects of purified anterior pituitary growth hormone on the response to water load and the daily water exchange in hypophysectomized dogs. Glomerular filtration rate and renal plasma flow were concomitantly determined in an effort to evaluate the role of these functions in the alterations in water

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metabolism produced by growth hormone.

Methods. Seven female, hypophysectomized dogs were used. The technic of hypophysectomy§,‖ and the after-care have been described in a previous publication(6). All animals used in this study exhibited maximal sensitivity to the hypoglycemic action of insulin thereby indicating that the hypophyses had been successfully removed(6,7). Thus far 2 of the animals (K-3 and K-8) used in this investigation have come to autopsy. Study of serial sections of a block consisting of a portion of the sphenoid bone, the tissue occupying the region of the sella turcica and the hypothalamus has revealed that neither adenohypophyseal cells (pars distalis, pars tuberalis and pars intermedia) nor cells of the pars nervosa were present in either of the animals(8). The remaining animals will be sacrificed and studied in a similar fashion at a later date. The technics employed for the measurement of renal function (glomerular filtration rate and renal plasma flow), urinary sodium concentration and the response to water load have been described in an earlier paper(5). The daily water exchange was determined by measurement of the 24 hour water intake and urine output. The animals were placed on a constant diet which was mixed with water(3,9). In addition water was provided *ad libitum*. Occasionally, there were periods (several days to 2 and 3 weeks) when the dogs consumed only a part or none of their basic food allotments and at such times, a diet of meat, fish, milk or eggs was substituted. The administration of growth hormone, however, was started only after the animals had been returned to and maintained on their standard diet for at least one week. The food allotment was not increased during the growth hormone administration. Growth hormone (Armour¶—Lot No. 22KR1) was given daily, intramuscularly, 1 mg/kg body weight for 14 to 33 days. In 2 experiments the dose of growth hormone was reduced after 7 days to 0.5 mg/kg. These doses of the hormone (same lot number) produced a marked anti-insulin and diabetogenic action in all hypophysectomized dogs studied(2,3).

Results. The effect of growth hormone was observed in 7 hypophysectomized dogs. Protocols of 2 representative dogs S-4 and E-4 are recorded in Tables I and II respectively.

Response to oral water loads. Before hypophysectomy the dogs excreted the orally administered water load (40 ml/kg) quantitatively in three hours(5,9). Following hypophysectomy the ability of all 7 dogs to excrete the standard water dose was considerably reduced. In dogs S-4 and E-4 the average excretions of the given water loads were 15 and 34%, respectively (Tables I and II). Gastric drainage revealed that no water remained in the stomach 40 minutes after the administration of the water load(5). During the growth hormone regimen the response to the water load progressively increased in every dog, reaching an excretion of at least 50 to 80% of the administered water. On one occasion dog S-4 excreted 109% of the load. After discontinuing growth hormone administration the excretion of the water load gradually declined to the range observed before the hormone regimen.

Daily water exchange. Within 24 hours following hypophysectomy the animals developed the usual transient diabetes insipidus which lasted from several days to 3 weeks. The severe polyuria gradually diminished and

§ Topical Thrombin, which was very helpful in controlling hemorrhage during the operation, was kindly supplied by Dr. A. C. Bratton, Jr. of Parke Davis and Co.

‖ We would like to thank Dr. R. K. Richards of Abbott Laboratories for a generous supply of sodium thiopental (Pentothal). The hypophysectomies were carried out under morphine-pentothal anesthesia.

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TABLE I. Effect of Growth Hormone on Water Exchange, Response to Water Load and Renal Functions in a Hypophysectomized Dog Having Mild Polyuria. Dog S-4.

Date	Status	Body wt, kg	Water exchange,* ml/24 hr: Water intake	Urine output	Urine sp. gr.	% water load excreted, 3 hr	GFR, ml/min.	RPF, ml/min.
6/13/50	Normal	15.8	700	450	1.028		66.8	320
16						113		
22		14.1	750	480	1.025		75.3	318
6/30/50	Hy-ectomy	14.2						
7/ 1-2			5135	4340				
20-27		15.8	2638	2054				
28			2560	1900			17.1	58
8/ 3-10			1457	1200†				
11			1800	1100†		14		
15			1240	1200†			15.7	61
9/ 7-13		15.2	1019	1020†				
14			1080	950†	1.010	16		
9/26 } 10/ 3 }		16.0	2234	1885	1.005			
10/ 4	Growth H.‡	16.0	1810	1875				
5	”		1980	2075	1.005			
7	”		3270	2625	1.003			
9	”	17.7	3250	2800	1.001	57		
10	”		4158	3815	1.002		26.3	102
12	”		5450	4820	1.001			
13	”		4770	4250	1.002	72		
15	”	18.0	6350	5130	1.002			
16	”		5400	4500	1.002		30.3	104
17	”	18.8	5810	4675	1.001	109		
26	”	17.9	4394	4480	1.002	81		
27	”	17.9	4990	4800	1.002		28.7	98
10/29			4370	3940	1.002			
30		17.8	4190	4270	1.002	68		
11/ 2		18.0	3240	3380	1.004	59		
4		17.3	2820	2830	1.004	57		
8		17.4	2350	2335	1.005	47		
9			2035	1920	1.004		16.3	63
11		17.6	2010	2560	1.004	53		
12-18		17.5	1835	1652	1.007			
12/29 } 1/ 5/51 }		18.4	2300	2060	1.006			

* When the time period is >1 day the water exchange figure represents a 24-hr average for the given period. In no case did the spread between successive days in the averaged periods exceed ±400 ml. GFR: Glomerular filtration rate (creatinine clearance). RPF: Renal plasma flow (p-aminohippurate clearance). Hy-ectomy: Hypophysectomy.

† Dog ate poorly.

‡Growth H.: Growth hormone, 1 mg/kg body wt daily.

the water exchange returned to within normal levels in 2 animals (*e.g.* the urine output of dog E-4 averaged 560 ml between 10/8/50 and 10/13/50), while in the others it remained at a level 2 to 3 times that found normally (*e.g.* the urine output of dog S-4 averaged 1885 ml between 9/26/50 and 10/3/50). The difference between these 2 groups was not due to variations in dietary intake since all animals had consumed their full basic diet for at least one week prior to growth hormone administration. Nevertheless dietary intake influences water exchange as evidenced by dog S-4 (Table I). Between 8/3/50 and 9/14/50 this dog ate poorly and consequently her urine output was approximately one-half that observed during her periods of full diet consumption (7/20-27 and 9/26-10/3/50).

Growth hormone administration resulted in a progressively increasing excretion of urine of low specific gravity containing neither sugar nor increased amounts of sodium chloride. The increased excretion of urine persisted

TABLE II. Effect of Growth Hormone on Water Exchange, Response to Water Load and Renal Functions in a Hypophysectomized Dog Having Normal Water Exchange. Dog E-4.

Date	Status	Body wt, kg	Water exchange,* ml/24 hr: Water intake	Urine output	Urine sp. gr.	% water load excreted, 3 hr	GFR, ml/min.	RPF, ml/min.
2/21/50	Hy-ectomy	15.0						
23-24			6305	5280				
27 / 3/ 5		15.3	2144	1794				
3/18-25		15.5	1371	895				
31 / 4/ 6		15.7	819	649				
5/22-28		16.4	750	616				
7/25		17.4				33		
10/8-20		18.9	835	560	1.015			
21			800	540			15.7	58
23			845	575		36		
10/24/50	Growth H.†	18.9	1236	900	1.009			
25	"	18.9	750	300	1.016			
26	"		900	450	1.015			
27	"		940	550	1.016			
28	"	19.3	1300	870	1.005	36		
29	"	19.8	1992	1540	1.004			
30	"	18.6	2060	1760	1.002		22.2	77
31	"	19.7	2440	2120	1.003	69		
11/ 1	"	19.8	2845	2155	1.002	79		
2	"	20.0	1990	1500	1.004			63
3	"	20.1	2080	1820	1.004		23.2	73
4	"	19.8	1770	1471	1.006	53		
5	"	19.6	2132	1955	1.005			
6	"	19.6	1370	1260	1.008		23.5	84
7	"	19.9	1530	1091	1.007			
11/ 8/50		19.7	957	725	1.008	50		
10		19.4	750	700	1.011		17.0	65
11		19.6	1080	979	1.010	49		
13-18			892	699	1.012			
12/29/50 / 1/ 5/51			905	532				
1/ 6/51		18.5	900	480	1.014	25		

* When the time period is >1 day the water exchange figure represents a 24-hr average for the given period. In no case did the spread between successive days in the averaged periods exceed ±400 ml. GFR: Glomerular filtration rate (creatinine clearance). RPF: Renal plasma flow (p-aminohippurate clearance). Hy-ectomy: Hypophysectomy.

† Growth H.: Growth hormone, 1 mg/kg body wt daily.

throughout the period of hormone treatment. In some animals the polyuria declined from the peak values while still on growth hormone, even though the animals were consuming their full basic diet (dog E-4). Several animals ate poorly during growth hormone administration and although their water exchange was reduced, it still remained well above the control levels. When the hormone injections were discontinued the urine excretion returned to the pre-hormone levels within one to 3 weeks.

Renal functions. In all animals after hypophysectomy there was a considerable decrease in the glomerular filtration rate and renal plasma flow. The filtration fractions did not change appreciably(4,5). During growth hormone treatment both functions increased, approximately in the same proportion. Full restoration of these functions to the pre-operative status was not achieved in any of the hypophysectomized dogs. The improvement of these renal functions prevailed throughout the growth hormone treatment. In 2 dogs some decline from the maximum levels was noted. White, Heinbecker and Rolf(10) observed full restoration of the renal dynamics during growth hormone ad-

ministration with approximately the same dosage of hormone. The discrepancy in results is perhaps due to the fact that there was a greater reduction of glomerular filtration and renal plasma flow rates in our dogs.

Discussion. The data just presented indicate that growth hormone administration to the hypophysectomized dog significantly improves its ability to excrete a water load and increases the level of water exchange. The growth hormone preparation used contained small amounts of adrenocorticotrophic and thyrotrophic hormones(3). However, the administration of ACTH in amounts 50 times that contained in the growth hormone preparation did not significantly alter the water exchange of hypophysectomized dogs. Very large doses of thyroxin did produce a severe diabetes insipidus in hypophysectomized dogs (11) along with signs of thyrotoxicity (*i.e.* persistent tachycardia and marked loss of weight). None of the growth hormone treated dogs exhibited any such signs. The observed effects on water metabolism, therefore, cannot be attributed to these two contaminants.

The mechanism by which growth hormone improves the ability to excrete a water load and increases the level of water exchange in the hypophysectomized dog is not yet clear. The diabetes insipidus produced by interruption of the hypothalamico-hypophyseal tract represents a hormonal deficit in that antidiuretic hormone (and possibly other posterior pituitary hormones) is absent while the anterior pituitary hormones are present and are indeed indispensable for the development of a severe permanent diabetes insipidus. According to Handley and Keller(12) the filtration rate, renal plasma flow and $Tm_{Glucose}$ are reduced in diabetes insipidus. However, White, Heinbecker and Rolf(13) indicate that the reduction in filtration rate, renal plasma flow and $Tm_{Diodrast}$ is only slight and transient when diabetes insipidus is produced without injury to the anterior hypophysis at the time of operation. In any case, the filtration rate in the diabetes insipidus dog is less affected than in the hypophysectomized animal. Further, the action of pitressin on water reabsorption by the tubules is independent of the filtration rate(14). In contrast, the hypophysectomized animal is deficient in all pituitary hormones and as a consequence develops atrophy of all the target glands. These animals manifest great reduction in filtration rate and renal plasma flow, severe impairment of all tubular functions, and inability to excrete a water load. The decreased filtration rate of the hypophysectomized dog appears to contribute to its impaired excretion of a water load and is in all probability a factor in limiting the diabetes insipidus expected in these animals on the basis of their deficiency of antidiuretic hormone.

The administration of growth hormone to hypophysectomized animals results in an increase in the filtration rate and renal plasma flow, improvement of tubular functions, return toward normal of the ability to excrete water loads and the production of a severe polyuria. The increased filtration rate induced by growth hormone appears to be responsible, at least in part, for the improved excretion of a water load. It may also be postulated that, in the absence of effective amounts of antidiuretic hormone, the increased filtration rate results then in severe polyuria. However, the changes in filtration rate and the ability to excrete a water load are not strictly parallel during growth hormone administration (*e.g.* dog E-4, Table II) and a direct hormonal effect on the renal tubular reabsorption of sodium and water cannot be excluded at present. The growth hormone induced polyuria in the hypophysectomized dog suggests that this hormone is one of the anterior pituitary principles the presence of which will permit the development of diabetes insipidus following interruption of the hypothalamico-hypophyseal tract.

The lack of growth hormone is shown to

10. White, H. L., Heinbecker, P., and Rolf, D., *Am. J. Physiol.*, 1949, v157, 47.

11. Unpublished observations.

12. Handley, A. C., and Keller, A. D., *Am. J. Physiol.*, 1950, v160, 321.

13. White, H. L., Heinbecker, P., and Rolf, D., *Am. J. Physiol.*, 1942, v136, 584.

14. Shannon, J. A., *J. Exp. Med.*, 1942, v75, 387.

be responsible for some of the disturbances in water metabolism found in the hypophysectomized dog. It may be a factor in the maintenance of normal water metabolism but probably not in the sense of a direct regulator as is the role given to antidiuretic hormone.

Summary and conclusions. Continued administration of purified growth hormone significantly improves the hypophysectomized dog's impaired response to water load. Concomitantly it increases the daily water exchange to levels seen in surgically induced diabetes insipidus. Growth hormone increases the glomerular filtration rate and renal plasma flow of hypophysectomized dogs but not to the pre-operative levels. It is suggested that growth hormone is one of the factors concerned with the maintenance of normal water metabolism.

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